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[GANN, 47, 105~116; June, 1956]

A HISTOLOGICAL STUDY OF SPONTANEOUS AND TRANSPLANTED MAMMARY TUMORS OCCURRING IN A NEWLY SEGREGATED HIGH MAMMARY CANCER STRAIN AND OTHER STRAINS OF INBRED MICE

(With Plates XVIII—XXIV)

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INTRODUCTION

Since 1947 we have been segregating a high mammary cancer strain of mice, and now, in 1955, this strain has reached the fourteenth generation. We have encountered three main difficulties in this process. First, in the spring of 1953, we began to use a synthetic diet which we thought was nutritionally sufficient. Following this diet change, we found that the mice did not conceive for about six months, or if pregnant, the pregnancy ended in a stillbirth or in the death of the mother postpartum. The causes of these occurrences are not clear at present. In addition to the lack of a littering factor in the food, the mice did not appear to like the diet and probably suffered from calory lack. Secondly, we had an epidemic of pneumonia in our mice toward the end of 1953, and we lost almost half of them. We thus came to have only a few colonies of mice toward the end of 1954, but we made brother-sister matings between these survivors. Lastly, the long-continued inbreeding resulted in a decrease of the offsprings in each gestation, a decrease in the frequency of conception, and in a decrease in the resistance to disease of the offspring.

Thus we experienced several unexpected difficulties in the segregation of this inbred strain. Nonetheless we did succeed in obtaining many spontaneous mammary cancers and we have classified them histologically.

MATERIALS

Up to the present time twenty-nine female mice, from age six to seventeen months (average of 10.3 months) have produced spontaneous tumors. All of the tumors occurred in the mammary glands and with two exceptions these female mice had littered. Of these two exceptions, one is a virgin and the other is an ovariectomized three month old female. The expected cancer developing age of both is ten months. We applied an olive oil solution of 20-methylcholanthrene to

the vaginas of ten female mice (unpublished data) and in two of these cancers of the breasts developed at the twelfth and thirteenth month respectively. We had, of course, expected uterine or vaginal cancer, but none developed. We do not know as yet whether the chemical in the vagina had any influence on the development of the breast cancer, or whether, which is more likely, they developed spontaneously.

In our histological studies, in addition to the tumors of our own strains of mice, we used spontaneously developed mammary cancers in mice which Dr. Toru Miyaji had obtained from the National Cancer Institute in the United States (34 cases of strain C 3 H/He, 2 of DAB2 and 20 of RF) and mice from our market stock.

Histological classification:

The difficulties in a histological classification of human mammary cancer are attributable to the heterogeneity sometimes encountered in the histological figures, the fact that even in the same tumor there is variation of these figures between one site and another, and that many of the figures represent transitional forms.

For these reasons Willis states that it is meaningless to make a detailed classification of mammary cancers and that it is sufficient to separate them into intraductal, extraductal and metaplastic types. On the other hand Dr. Fred Stewart proposes the following, elaborate classification: 1. Paget's disease, 2. Carcinoma of Mammary Ducts, 3. Carcinoma of Lobules, 4. Rare types, 5. Sarcomas or sarcoma-like tumors. The classification of the tumors in mice differs somewhat from that in human beings.

The first investigator to classify mammary cancer in mice was Apolant, and recently Cloudman, Dunn and others have made similar efforts, but none of them has been satisfactory.

In our series almost all of the mouse mammary cancer originated from duct or gland epithelium, and if we exclude a few non-epithelial tumors, all of our cancers may be called adenocarcinoma. However, I consider it proper that tumors arising from ductal epithelium should be separated in the classification from those arising from acinar cells. Hence I classify these tumors as ductal and acinar types, and, in addition, I add the metaplastic, or cornified type, calling this latter an "adenoacanthoma". My final, or miscellaneous group includes non-epithelial tumors.

Andervont et al. state that as the relationship between the biological attitude of the tumor and its histological picture are not yet clear, and since sometimes tumors with a benign microscopical appearance show a rapid growth or metastasis after transplantation, therefore the distinction between benign tumors and malignant tumors is quite meaningless. I, however, continue to feel that from the standpoint of histological embryology it is worth while to differentiate those

tumors arising in acinar cells from those arising from ductal cells.

1. Ductal Type: (Fig. 1 to 4)

This type of tumor is thought to arise chiefly from the ductal or emunctory epithelium. Abundant and thick stroma surrounds the large tumor cells (Fig. 1). The tumor cells usually swarm to form dense masses. In the periphery they are columnar or cuboidal and in the center they are generally polygonal. There appears to be a gradual change between the two forms. The nuclei are round or ovoid, rather large, and many of them are pyknotic. They are centrally placed, and compared with the other tumor types they have many mitotic figures. The cytoplasm is basophilic and scanty (Fig. 2).

The following sequence of events appears to take place in the development of this tumor: The ductal epithelial cells grow in an abnormal manner to gradually fill the lumen (Fig. 3 and 4) and a cribriform or lacy pattern results. A hemorrhage occurs in the obstructed duct and if necrosis occurs among the abnormal epithelial cells, the appearance of a "comedo" form of tumor is attained. Around these tumors in the ducts there are elongated, thin cells that resemble the basal cells of the normal duct epithelium. In a well-differentiated tumor these cells can be recognized lying between each affected duct. These cells are elongated because they are compressed between neighboring cancer cell nests as these gradually increase in size. These cells (stromal?) are always found surrounding the tumor cell groups, but never between the tumor cells. This is one point of difference between this type of tumor and the acinar type (Fig. 2).

Some of the tumors of this type have a pattern which suggests that they should be called adenoma rather than carcinoma. And some, with a large (central) lumen, we have called "cystomas." However, we use this latter term only as a qualifying adjective, such as cribriform or comedoform. All of these tumors have a homogeneous substance which can be stained pink with eosin. The narrower the lumen of the affected duct becomes, the more this tumor resembles that of the acinar type. This is probably due to the accumulation of secretory products caused by the more distal obstruction of the duct. The basis on which we differentiate this type of tumor from that of the acinar type is that masses of cells project from the neoplastic mass in the duct wall toward the lumen of the duct, and therefore we feel it reasonable to assume that the origin of the tumor is from the duct wall. It is known that the ductal epithelial cells of the mammary glands have the embryological potential of differentiating into glandular cells.

2. Acinar Type (Fig. 7, 8)

In many ways this tumor resembles that of the ductal type. However, the tumor cells generally make an acinar architecture and fragile collagenous fibers intrude among the cells at their periphery. The tumor cells are quite similar to

normal glandular cells in the mammary gland. The oval nuclei rich in chromatin, occupy a basal position. Mitoses are rarely seen. The cytoplasm is slightly acidophilic in its luminal side and basophilic near the nucleus. The lumina of the acini are very small, or often are absent. The stroma is scanty but fine stromal fibers accompany capillaries into smaller ramifications of the tumor than in the ductal type. Secondary changes, such as hemorrhage and necrosis, are more frequent in the stroma than in the parenchyma. It was of particular interest to us that all of the mice thaf were pregnant and developed a tumor, had this type of tumor.

3. Metaplastic type (Fig. 9)

Tumor itself may sometimes cause metaplasia. In this classification, however, I confine the use of the term to a cornifying tumor or to one which has squamous metaplasia and in the human case would be called "adenoacanthoma". This type of tumor is not thought of as a tumor of squamous cells, but rather as an epidermidization of a tumor originating in glandular epithelium. Andervont and Kirschbaum used D.B.A. strain and Heston the C_3H/b strain and they found these metaplastic type tumors in many cases in which the young had not been nursed by the mother, but on the other hand when they were dealing with a mouse that had been breast fed they encountered adenocarcinoma.

4. Sarcoma (Fig. 10)

This type is quite rare among spontaneously occurring tumors. In my series this tumor was found with epithelial mammary cancer in the mammary glands of mice in the uteri of which we had repeatedly applied 20-methylcholanthrene. The histological picture was that of fibroblasts and atypical fibroblasts.

Results and The Consideration Thereof

1. The strains of the mice and the frequency of development of the several types of tumors (Table 1):

Table 1. The strains of the mice and the frequency of development of the several types of tumors.

	RF	C3H/He	DBA2	C3H/N	Our's	Stock
Ductal	15	19	1	4	35	9
Acinar	8	23	1	1	11	4
Adenoacanthoma	1	1	0	0	1	0
Sarcoma	0	0	0	0	2	0
Total	24	43	2	5	49	13
No. of Mice	20	34	2	4	30	13
Tumor / Mice	1.26	1.26	1.00	1.25	1.63	1.00

The acinar type of tumor was frequent in the C 3 H/He strain and the ductal type in other strains. This was a clear-cut finding in my series. The fact that we list more tumors than there were mice is explained by the multiplicity of tumors in some mice. In my own strain the average number of tumors was 1.63, and the averagae that I found with C 3 H/He was 1.26 and with R. F. strain it was 1.20. I found that the ductal type occurred most frequently in my strain.

2. Relation of the incidence of tumor type and the cancer age of the mice:

It is said that in the DBA strain the acinar type of tumor is more often found in old mice, and a solid, cellular, proliferating mass is characteristic of the tumors in the young mice. I have noted the same tendency in my strain. Below (Table 2) I have classified the type of tumor as related to the age of occurred cancer.

Table 2. Type of tumor and the incidence of age.

Age of animals (months)	6-11	12-17	over 18
Ductal type	18	8	1
Acinar type	5	4	0

The incidence of the ductal type tumor is higher in the sixth to the eleventh month than in the twelfth to the seventeenth month, but the incidence of acinar type tumors is about the same in both of these age groups. One result of this is that the acinar tumors are the predominant type in the older age group and the ductal tumors are predominant in the younger group.

3. The tumor type related to the influence of hormones:

The development of cancer was found in one virginal, and in one ovarietomitized mouse. The virginal mouse had been separated from the males from the weaning period. The other mouse had been sterilized a month after birth by removal of the ovaries. Both had the development of their tumor ten months after birth. A ductal tumor was seen in the virgin and an acinar tumor was seen in the gelded mouse. (Fig. 15)

Four acinar tumors were noted wherein conception occurred after the tumor was noted. Two were in mice of the RF strain and two were in my strain (Figs. 13 and 14).

According to Gardner and Strong, theelin cause the development of the ductal system of the mammary glands and lutein hormone multiplies the peripheral parts. Therefore, the fact that mammary cancer in mice during pregnancy has an acinar structure is due to the balance of hormones at that time. The incidence of mammary cancer in the C3H strain and A strain was almost the same in mice who had littered but in virgins the incidence in these two strains was different. In the C3H strain the incidence in the virgin was the same as in the post-partum mouse, whereas in the A strain only 5 % as many virgins had cancer as occurred

in the post-partum group. The A3CHF$_1$ strain that was obtained by mating the above two strains showed 91 % development of cancer in the virgin mice. Of course many factors, including heredity, the influence of hormones and the milk factor, have their part in the development of cancer in the mouse and we can not postulate the whole mechanism on the basis of a small series. However we feel safe in assuming that lutein as well as estrogen hormones are related to the development of mammary cancer in the mouse, because in the C3H strain the incidence of mammary cancer is high even in the virgin, and the predominant cancer in this strain is the acinar type, which type is seen in mice in general usually during pregnancy.

4. The type of tumor and the metastses:

Of the mice of my strain, nine showed metastases when we autopsied them, and all of these had lung metastases. Other organs involved to various degrees were the liver, spleen and heart (Table 3).

Table 3. Type of tumor and the organs having metastasis.

Histological type	No. of animal	Metastasis to			
		Lung	Spleen	Liver	Heart
Ductal type	7	7	1	1	2
Acinar type	2	2	1	1	0
Ductal and Acinar type	1	1	0	0	0

In one case of metatasis to the heart an intraventricular embolus was found, presumably due to the intrusion of tumor cells into the blood supply at the time of the removal of the breast tumor, and in another case the tumor had extended into the heart from an adjacent lung metastasis. In the liver the metastases were seen around portal vein branches and in the spleen in the area said to be the center of the embryo bud. It appears that both of these types of metastases were blood-borne. The relation of metastases to the primary tumor is shown in table 3.

A case of the metaplastic type merits special mention. It was cornified at first, but gradually lost this appearance and turned into a solid, proliferating mass. Histologically, it was of the ductal type. In another case there were two tumors, one of acinar and one of ductal type. The metastasis in the lung was of the ductal type. Of thirty-five animals that had ductal type tumors, six had metastases, and of eleven mice with acinar tumors, one had metastases. It, therefore, appears that ductal tumors are more likely to metastasize.

5. Tumor age and Metastasis:

The following table shows the relation of tumor age to metastases in nineteen mice of my strain, autopsied at the months indicated.

Tumor age:	one month	two months	three months
mice with metastases	3	2	4
mice without metastases	6	3	1

This brief series only suggests that the mice which lived longer had more metastases than those who died at an early age.

(a) Extirpation of the tumor as related to metastasis:

	Number of animals with metastasis	Number of animals without metastasis
No treatment	6	9
Extirpation of primary tumor	3	1

Thus it is seen that the rate of metastasis in the animals which had been operated upon is far higher than the rate in the non-operated animals. However, the life of the animals was lengthened by the operation and they thus had more opportunity to develop metastasis.

(b) The relation of age of spontaneous death to the factor of treatment or non-treatment:

Tumor age:	1 month	2 months	3 months
Non-treated	8	5	2
Extirpated	1	0	3

It is obvious that the extirpation has made the life of the animal, or the tumor age, longer, and, as mentioned above, this is probably related to the increasing number of metastases seen in the treated animals.

6. The type of recurrence seen after extirpation:

In the nine animals in which operation was performed, seven had ductal type tumors, one was acinar in type, and one was metaplastic.

In three of the seven original ductal tumors, the recurrence had the same solid cellular mass form of the primary tumor and another four recurred with the primitive cystic form which was seen in their primary tumors, but the cysts in the recurrent tumors were smaller than in the primaries. The acinar tumor assumed the ductal form in its recurrence. The metaplastic tumor lost its cornified element in its recurrence but still formed cysts. A second extirpation in this case was followed by another recurrence and this was of the pure cystic variety. It appears that in recurrences of acinar and metaplastic tumors the ductal pattern

eventually prevails. However, we are not sure whether this is related to extirpation or whether the original tumor is differentiating in the direction of the ductal type anyway. And there is the third possibility that a tumor with ductal origin develops from neighboring mammary tissue after the extirpation.

7. The tumor type as related to its transplantability:

I have already stated that the transplantability of spontaneous mammary cancer in mice stays within the genetic barrier. We attempted to transplant four tumors of the acinar type and with one there was a take. We also tried with five of the ductal type and had one take, and we tried one metaplastic type tumor without success. Compatibility or non-compatibility does not appear to be related to the tumor type.

8. Successive transplantations and their histology:

We made successive transplantations of spontaneous mammary cancer of MK-7 during two years, and during this time the scope of its transplantibilty and the agressiveness of its histological picture both increased. The length of life of the host varied inversely with the number of the transplant while the number of metastases found was increasing. The histology varied as follows: MK-7, which at first was ductal and a small cystic adenocarcinoma (Fig. 17, 18) showed a more rapid proliferation of ductal cells which filled the lumina more and more as the generations of the tumor increased and finally changed into an expansive, solid state (Figs. 19, 20). In the center of these large masses of tumor cells necrosis began to be seen. This change was noted from the seventh generation and became remarkable from the eleventh generation, at which time it often assumed the appearance of "comedo carcinoma". After the twenty-third generation there was a lowering of the transplantibility and naturally healing cases of the tumor increased. After the twenty-fourth generation there was no further take.

Our experience is therefore different than that with the Ehrlich cancer in which successive transplantation cancer in the mammary glands comes to show a non-epithelial histological picture. It may be that the interstitial tissue in the above case contained a tumor factor when the tumor was transplanted, and so developed in the new host. In my own cases I do not believe that there is any evidence of a stromal tumor factor, and as a matter of fact the stroma could not keep pace with the growth of the neoplastic epithelial cells. Consequently the nourishment of the tumor was gradually interrupted and the potential of the tumor gradually destroyed.

It is interesting to observe tumor which has been transplanted under the skin and to correlate its appearance with its age. In such tumors the cytoplasm and nuclei expand and do not take staining for the first two or three days. It seems almost as if they were necrotic. On about the seventh day one observes at the

periphery a growth of new cells with basophilic cytoplasm and small nuclei full of chromatin. On the fourteenth day the tumor cells begin to invade in a linear manner, sometimes along the lymphatic spaces and at other times along the tissue pockets which were formed at the transplantation (Fig. 25, 26). After this each mass of tumor cells assumes the configuration that it had before transplantation.

We made a first hybrid by mating our strain of mice with the C3H strain, and a second hybrid by mating the offspring of the first hybrid, and a cross by mating a C3H with the first hybrid. Then we transplanted MK-7 under the skin of each of these mice and there were seven takes in the first hybrid and twelve in the second. In the five mice got by the cross mating there was no take. The histological figure of the transplanted tumor in hybrids mated with another strain of mice showed the original solid type of tumor.

Conclusions

I have segregated a high mammary cancer strain of mice by continual brother-sister mating since 1947. I have made a study of the histology of spontaneous mammary cancer in this strain of mice and have separated four groups of adenocarcinoma, viz. ductal type, acinar type, the metaplastic type which is the result of metaplasia of the above two, and lastly sarcoma, which incudes all of the non-epithelial neoplasms in my classification.

I have also observed mammary tumors in the RF, C3H/He, DBA2 and C3H/N strains and have noted that in the C3H/He strain most of the tumors are of the acinar type, while in my own strain most were of the ductal type.

The ductal type was seen more frequently in young animals and the acinar type more frequently in older animals. When an animal with a tumor already established becomes pregnant, the tumor turns into the acinar type and this is probably the result of the influence of hormones. The rate of metastasis rises in direct proportion to the tumor age (which is the period from the development of a tumor until the death of the animal), and there are slightly more metastases with the acinar type.

Tumors of the acinar type often show the histological configuration of the ductal type in their metastases, and many of the tumors that recurred after extirpation belong to the ductal type. With continuing transplantation they lose the acinar quality completely.

From these data we conclude that the ductal type tumor has a lower level of differentiation and a higher growth potential than the acinar type.

Both the ductal and acinar tumors can be transplanted but are limited by genetic factors in their possibilities of take. That is, neither tumor has in itself, the ability to grow beyond the limitation of the histocompatibily of genetic factors.

MK-7, a transplantable mammary carcinoma was passed through twenty-five generations, at which time the transplants had developed a marked tendency to necrose, and the tumor was no longer transplantable. Mention was made of the probable function of the stroma relative to this developing tendency to necrose in the transplants.

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Fig. 1. Ductal type. Shows a relatively small lumen and as a whole reveals growth of solid mass (MK-6).

Fig. 2. High magnification of Fig. 1. Tumor cells are cuboidal, containing poor chromatin nuclei. Some cells are small and basophilic. (MK-6)

Fig. 3. Ductal type. Crowth of the duct-cells toward its lumen. The lumen remains and contains small amounts of the products. (MK-9)

Fig. 4. Ductal type (MK-8). Forming solid nests and have expansive growths.

Fig. 5. Ductal type (MK-8). The type of a growth of ductal cell. Cyst formation and is filled with blood.

Fig. 6 Ductal type (MK 1 A). A type of papillary growth.

Fig. 7 Acinar type (MK-31). Tumor cells are acidophilic, large cytoplasm with small

basal nuclei are arranged uniformly small and glandular.

Fig. 8 High magnification of the acinar type (MK-38). Small dark, round nuclei and large eosinophilic cells are noted. The acinar pattern is well-preserved.

Fig. 9. Metaplastic type (MK-48). Proliferation of the ductal cells is prominent without leaving the lumen. Beginning of cornification in the central portion.

Fig. 10. Sarcoma. Fibroplastic, non-epithelial tumor, which is induced with methylcholanthrene.

Fig. 11. Tumor of the virgin female mice. The growth consists of ductal cells with soild nests formation.

Fig. 12. High magnification of Fig. 11. The peripheral cells of the nest are cuboidal and the central area is polygonal and partially necrotic.

Fig. 13. Mammary carcinoma in the pregnant RF mice. The pattern is acinar.

Fig. 14. High magnigfication of Fig. 13. Shows acinar arrangement of acinar cells surrounded by delicate and scanty fibrous tissue.

Fig. 15. Mammary carcinoma of ovariectomized mice. The stroma is edematous.

Fig. 16. Induced carcinoma with vaginal painting of methylchoranthrene olive oil solution. The pattern of the growth is intraductal.

Fig. 17 and 18. MK-7. Ductal cell type. This tumor maintained serial transplantation during 25 generations. The figures reveal expansive growth of ductal cell and cellular infiltration in peripheral area. Many elongated cells due to pressure of cells are seen. Junior cells consist of dark nuclei and are polygonal with moderate anaplasia.

Fig. 19. Recurrent tumor after extirpation. The appearance is more pleomorphic and has more infiltrative tendency than the original tumor.

Fig. 20. Different area of the same tumor reveals ductal cell growth.

Fig. 21. Transplantable tumor (MK-7). At the 11th serial transplantation the metastasis is found in the lung.

Fig. 22. The same animal has a tumor which shows solid nest without luminal formation. In the stroma, there is massive hemorrhage.

Fig 23. The tumor which is the 15th generation of serial transplantation shows soild nests. The peripheral cells are cuboidal and have hyperchromatic nuclei. In the central area necrosis is noted.

Fig. 24. The 13th serial transplantation. One week after the implantation. The transplant shows almost complete necrosis with hemorrhage. In the peripheral zone small numbers of surviving tumor cells are noted.

Fig. 25 and 26. Two weeks after serial transplantation of the 13th generation. Beginning of a growth of a sarcomatous neoplasm. A cut section of a cut-gut is noted in the middle lower areas.

要　旨

新しく分離した乳癌好発系ハツカネズミ及び他系ハツカネズミ乳腺腫瘍の組織学的並びに転移による研究

赤松保之

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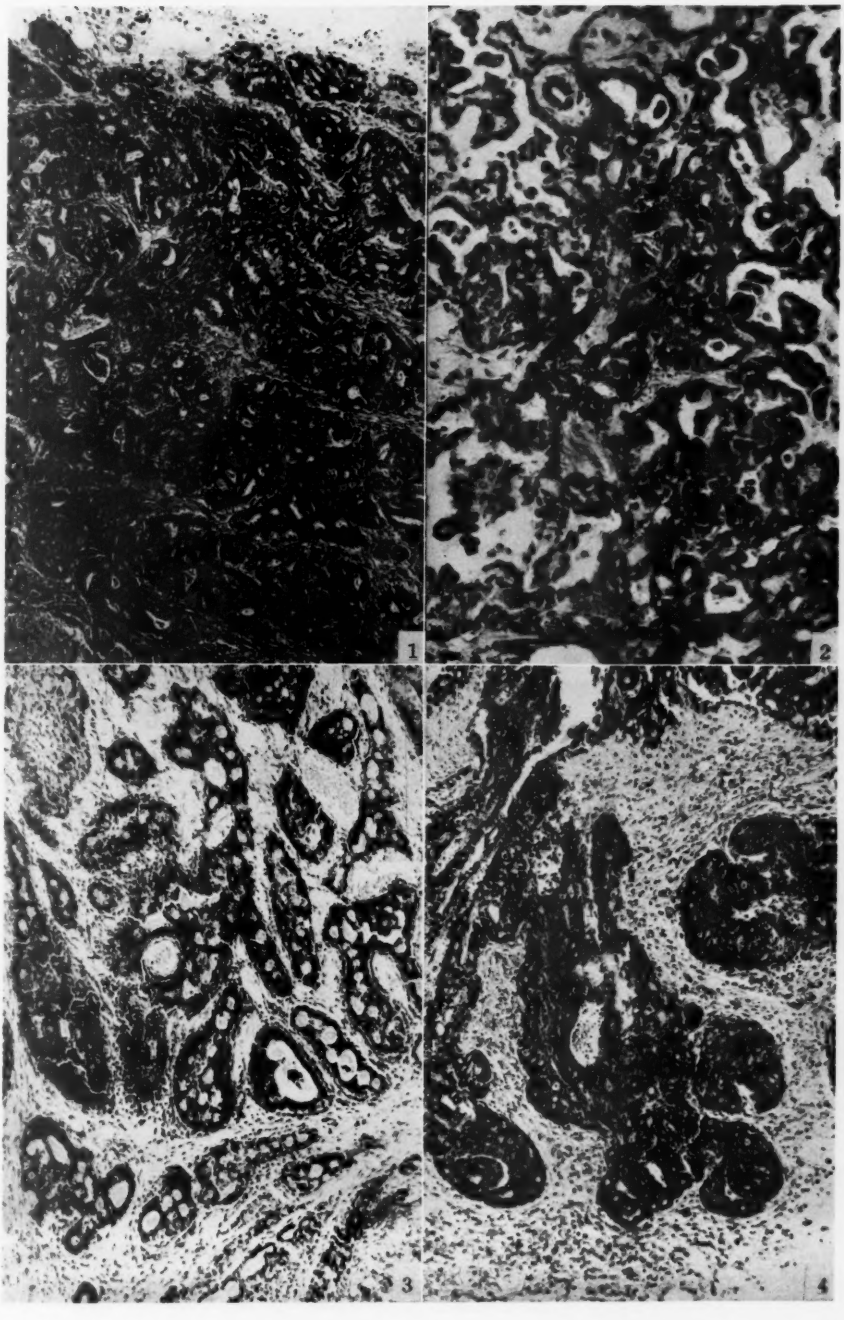
著者は，1947 年以来，兄妹交配をつづけて新しく乳癌好発系ハツカネズミを分離した。この系に自然発生した乳腺腫瘍を組織学的に検索して，主に乳管から発生した乳管型，腺房から発生した腺房型，後者から化生をおこした化生型などの癌に分類し，これらに非上皮性腫瘍としての肉腫を加えた。

この分類をもって，著者の系，RF，C3H/He，DBA 2，C3H/N 及び市販雑系ハツカネズミの腫乳腺腫瘍を比較すると，C3H/He 系では腺房型が多く，著者の系では乳管系が多いのがめだった。

また，幼若な動物には乳管系が多く，老年動物では腺房型が多い。担腫瘍動物が妊娠すると腺房型となることが多い。転移は，腫瘍年齢 (腫瘍が発生してから動物が死亡するまでの期間) に比例して高くなる。そして，腺房型に比べると，乳管系の転移率はやや高い。腺房型の腫瘍でも転移巣では乳管型を示す傾向があり，腫瘍摘出後に再発した腫瘍も乳管型が多い。また，移植をつづけると腺房系の要素が失われる。こうしたことから，乳管型腫瘍は分化の程度が低く，増殖力がつよいと考えられる。

移植は，乳管型，腺房型のいずれでもできるが，そのつくか，つかないかは遺伝的関係によって左右され，組織適合性 (histocompatibility) の限界をこえることはない。

著者の系からえた移植性腫瘍 MK-7 は，25 代にわたって継代移植できたが，ついに腫瘍細胞の壊死によって移植性を失った。この組織像から，間質のもつ腫瘍増殖に関する役割に言及した。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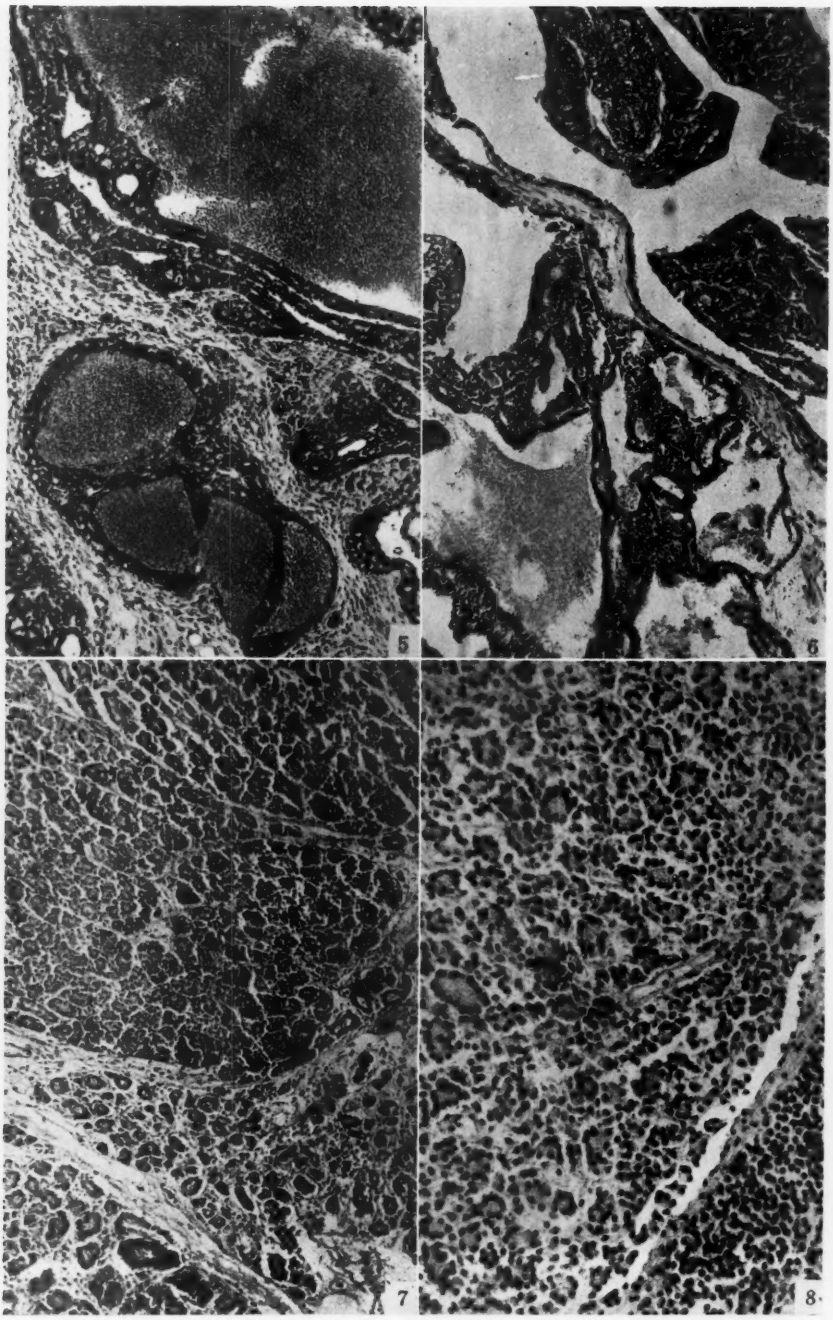
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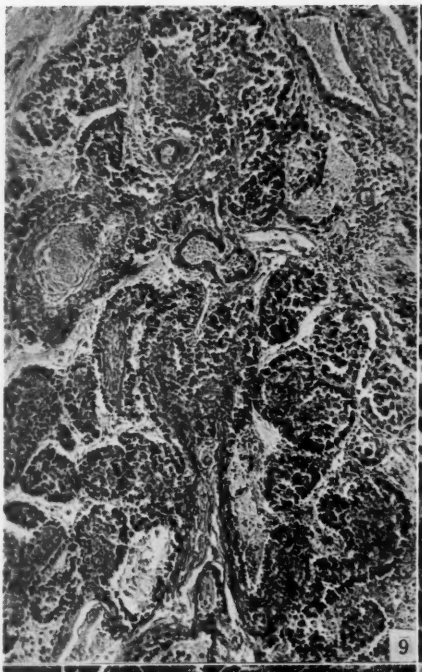
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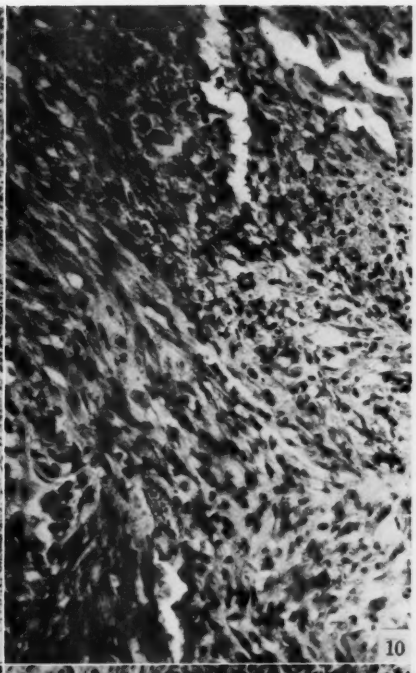
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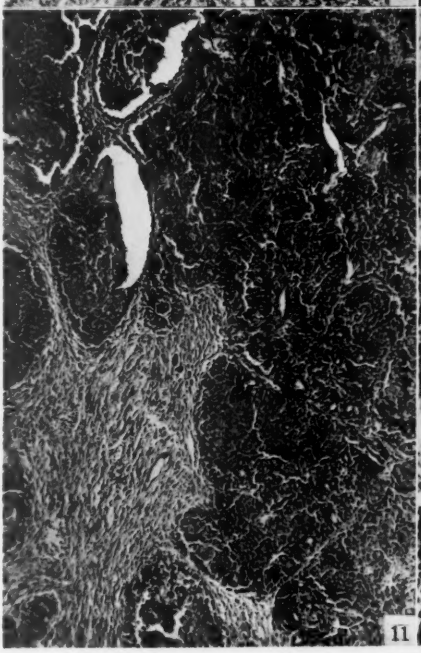
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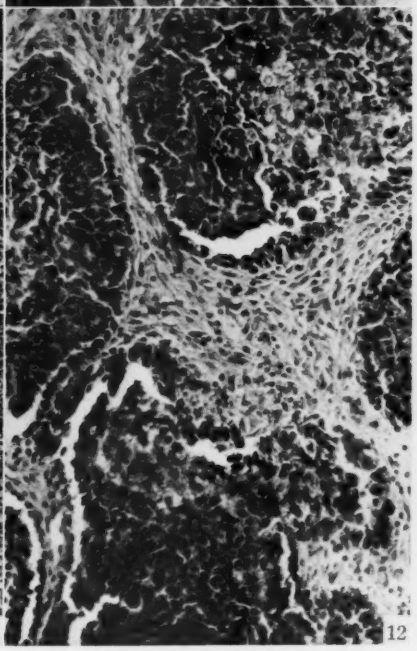
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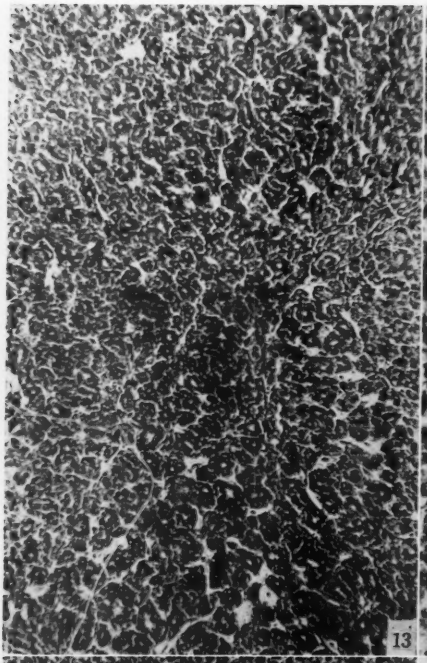
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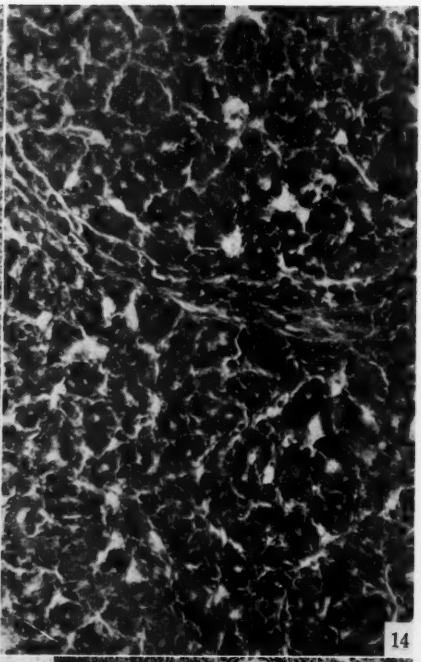
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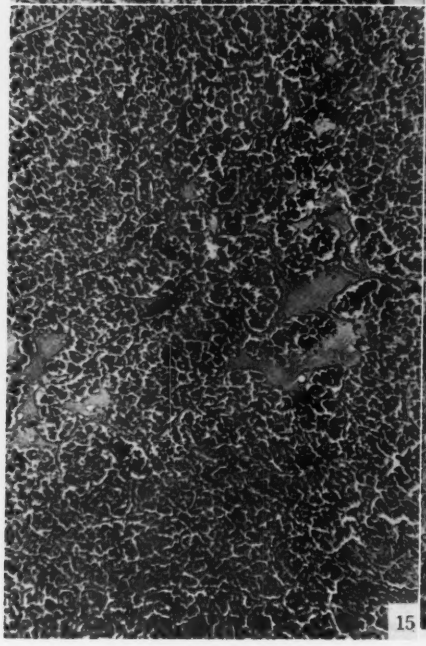
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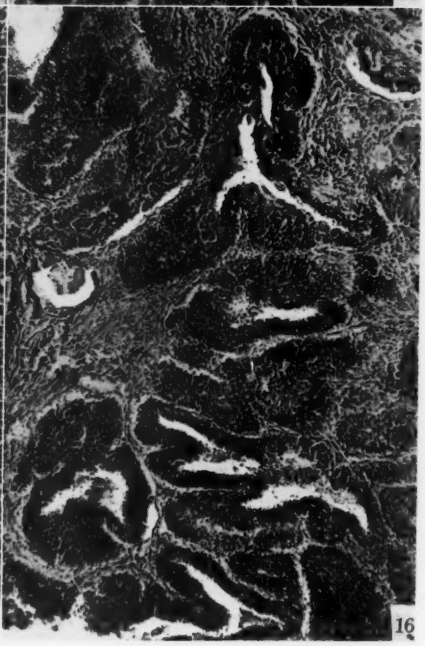
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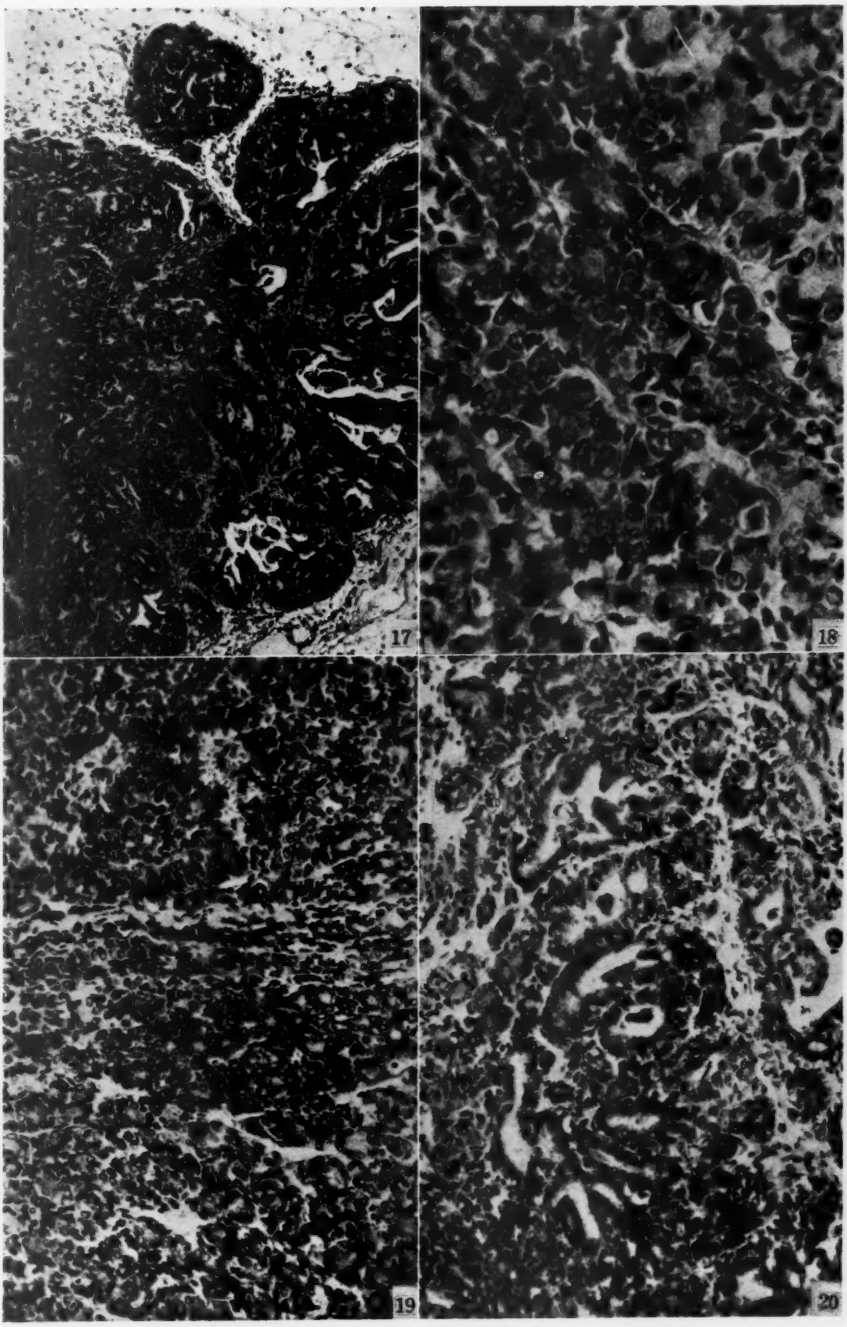
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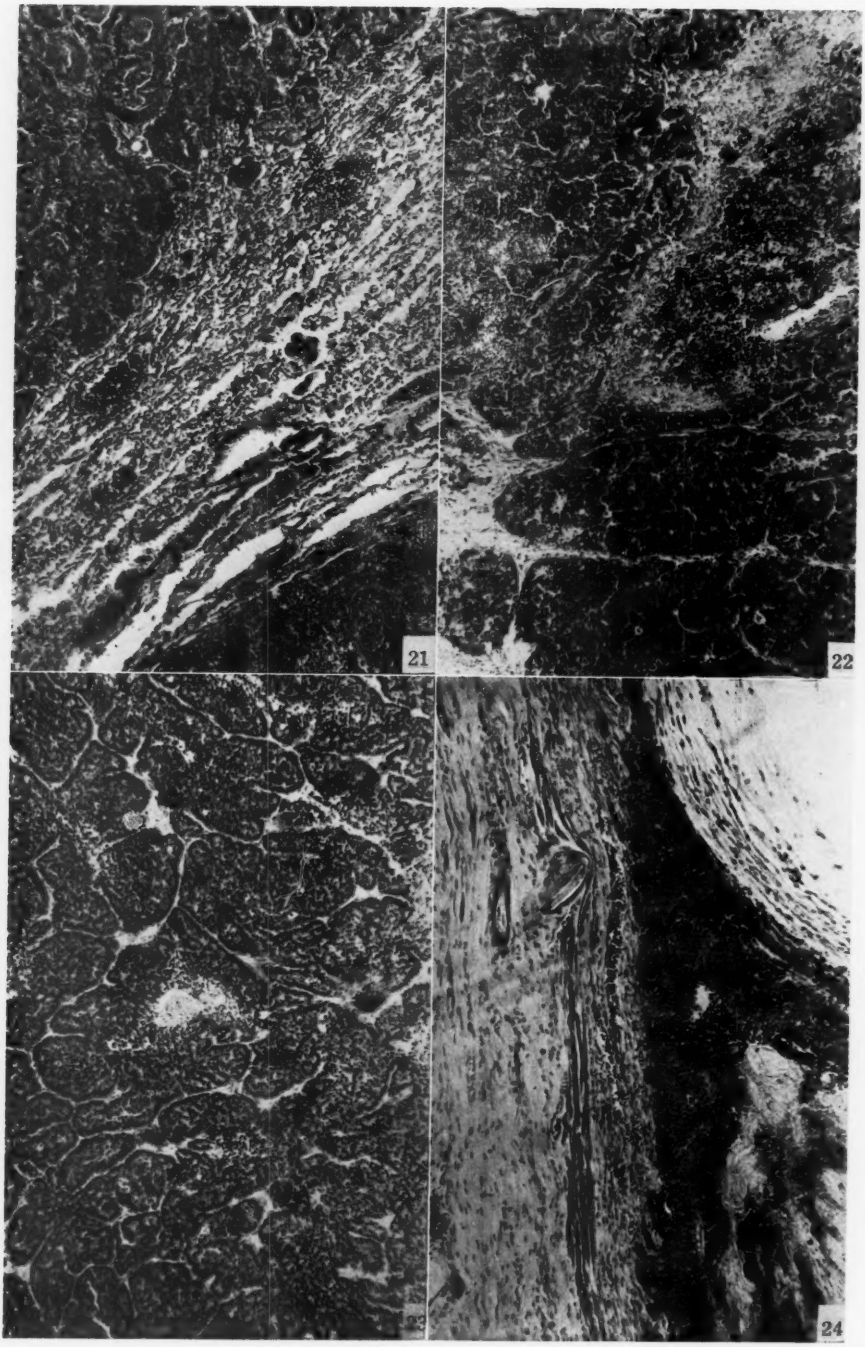
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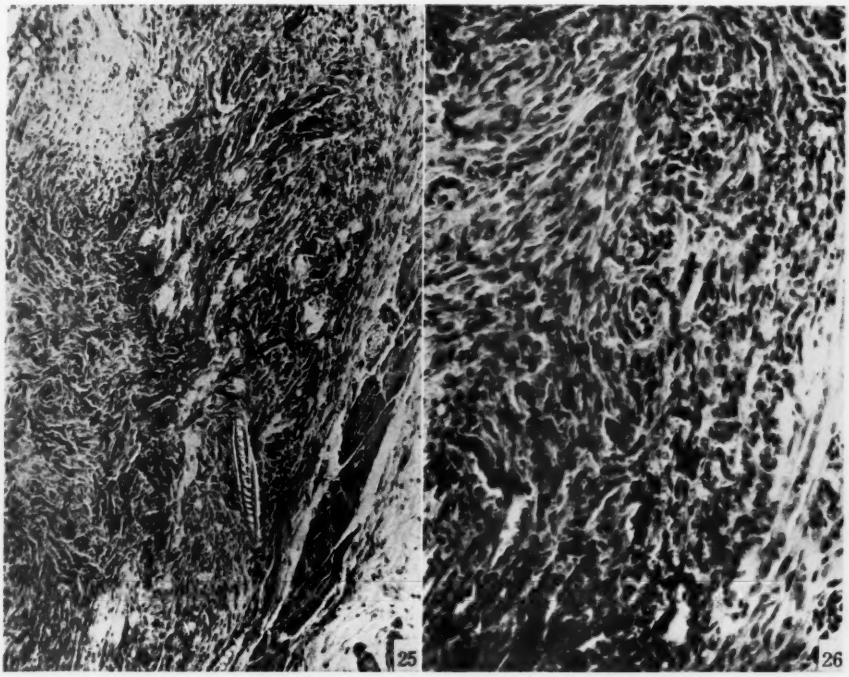

[GANN, 47, 117~128; June, 1956]

THE HIROSAKI SARCOMA. A LYMPHOSARCOMATOSIS OF THE RAT (With Plates XXV—XXVIII)

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The Yoshida sarcoma, which was established by Yoshida (1) in 1943 as an ascites sarcoma of the rat, has contributed greatly to the tumor pathology, but opinion is divided on the mother cell of the tumor. Yoshida concidered at first that the tumor might be a reticulosarcoma, but later he took the opinion that the mother cell of the tumor might be a "monocyte" after Amano (2). Tagashira and others (3) described that the leucaemic blood picture of the Yoshida sarcoma corresponds to that of human monocytic leucaemia. Hamazaki (4) took the opinion that the mother cell of the tumor might be a surface epithelium of the peritoneal cavity.

The Hirosaki sarcoma (5, 6, 7, 8) is a new ascites sarcoma of the rat which was established in the authors' laboratory by means of the inoculation of the orginal tumor into the abdominal cavity. The tumor is very similar to the Yoshida sarcoma and it has been clarified by our experiments that this tumor as well as the Yoshida sarcoma should belong to a type of lymphosarcomatosis.

On The Original Tumor

The Hirosaki sarcoma was established as an ascites sarcoma in 1951 by means of the inoculation of the original tumor into the abdominal cavity, which developed spontaneously in the cervical lymph nodes of a male hybrid rat. The remaining tumor of the original rat increased in size gradually, but no metastasis of the tumor was found in any organ in the postmortem examination two weeks after the excision (Fig. 1).

The orginal tumor was circumscribed sharply against the neighbouring tissues and showed in the histological examination a diffuse proliferation of large and clear tumor cells with a slight rest of the affected lymph node which was considered to be the orginal tissue (Fig. 2).

Cytological and Patho-Anatomical Findings in the Successive Intraperitoneal Transplantations

The tumor has been successively trasplanted intraperitoneally through 340

generations over 4 years, chiefly into hybrid rats and partly into Wistar rats.

I. Cytological findings:

The tumor cells in the ascites are round and stained basophilic by Giemsa's staining with a round or lobulated nucleus eccentric in the protoplasm. Most of the cells have a diameter or 25–35μ in smear preparation. Several fat vacuoles are usually found in the marginal part of the protoplasm. Lace-like characteristic vacuoles are found in this part usually on the day after the transplantation. One

Table 1. Metastases in various organs of rats which died of the tumor at various intervals after the intraperitoneal transplantation. (Diploid type).

Rat No.	Sex	Body Weight (g)	Survival Days	Peripheral Blood	Lymph Nodes									Heart	Lungs	Liver	Spleen	Kidneys	Bone Marrow	Pleural Cavity
					Cervical	Axillary (r.)	Axillary (l.)	Inguinal (r.)	Inguinal (l.)	Mediastinal	Perigastric	Retroperitoneal	Mesenteric							
754	♂	120	10	++++	−	++	−	++	±	++++	++++	++++	++++	++	++	++	++++	++	+	++++
756	♀	85	11	+	++	++	+	++	−	++++	++++	++++	++++	++++	++++	++	++++	++	+	++++
757	♂	115	6	+	−	−	−	−	−	+	++	++++	++++	±	±	±	±	±	±	−
758	♂	75	10	+	−	−	−	−	−	++	++	++	±	±	±	±	+	±	−	−
759	♀	75	15	++	++	++++	++++	++++	++	++++	++++	++++	++++	++++	++	+	++	++++	+	++++
763	♀	80	5	++	−	++	−	−	−	−	++	++	+	+	±	±	±	−	±	−
764	♀	150	8	++	−	−	−	−	−	++	++++	++++	++	±	++	±	±	−	+	++++
765	♂	180	5	++	−	−	−	−	−	+	+	++	++	+	+	+	+	±	+	++++
766	♂	100	11	++	−	−	−	−	−	++++	++++	++++	++++	+	+	+	+	±	+	++++
767	♂	160	12	++	−	−	−	++	−	++++	++++	++++	++++	+	++	+	++	+	+	++++
768	♀	100	10	+	−	++	++++	+	−	++++	++++	++++	++	+	+	+	++	+	±	++++
771	♂	100	10	+	−	−	−	−	−	++++	++	++++	−	+	−	−	+	−	±	−
772	♀	70	7	−	−	−	−	−	−	+	+	−	−	−	±	−	−	−	−	−
773	♀	100	14	++	−	++++	++++	++++	++++	++++	++++	++++	++++	±	++	+	−	+	++	++++
775	♂	90	17	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
776	♀	80	12	++	−	++	−	++	−	++++	++++	++++	++	±	+	++	+	±	+	++++
778	♂	80	9	+	−	−	−	+	−	+	++	++	++	±	±	±	−	−	−	−
779	♀	85	7	+	−	−	−	−	−	+	++	++	++	±	+	+	+	−	−	++++
780	♂	80	5	++	−	−	−	−	−	++	++	++	++	+	+	±	+	±	+	++++
781	♂	80	11	++	−	++	+	++	−	++++	++++	++++	++++	++	++	+	++	++	+	++++
783	♂	70	10	++	+	−	+	+	+	++++	++++	++++	++++	+	++	+	++	+	+	++++
784	♀	80	14	++	+	++++	++	++++	++	++++	++++	++++	++++	+	++	+	+	+	+	++++
786	♀	65	10	++	−	++++	−	++++	±	++++	++++	++++	++++	+	+	+	++	+	+	++++
792	♀	85	8	+	−	+	−	±	−	++	++	++	++	±	±	±	+	−	±	−
793	♀	80	8	+	−	−	−	−	−	±	+	++	+	+	±	+	+	±	±	++++
797	♀	100	6	+	−	−	−	−	−	++	+	+	++	±	±	±	+	±	±	++++
806	♂	85	5	+	−	−	−	−	−	+	++	++	++	±	+	±	±	±	±	++++

or several large nucleolei are usually found in the nucleus. Azur granules are often found in the protoplasm (Fig. 3). Supravital staining using neutral red and Janus green shows usually a rosette formation of neutral red vacuoles and dispersed Janus green granules in the protoplasm of the tumor cells.

Phagocytosis of carbon particles and peroxidase reaction were always negative (Fig. 4).

Chromosome number of the tumor cells varied showing a normal curve with the peak of about 37–38.

These cytological findings of the Hirosaki sarcoma are well in accord with those of the Yoshida sarcoma.

II. Patho-anatomical findings:

When the proliferation of the tumor was moderate, most of the intraperitoneally transplanted rats died of tumor in about 2 weeks with abundant milky ascites (tumor type), while when the proliferation was marked, most of the rats died in about 4–8 days with abundant haemorrhagic ascites (haemorrhagic type).

Postmortem examinations revealed marked infiltration of tumor cells into many organs macroscopically or microscopically corresponding to the duration of the course (Fig. 5). Most of the nodular tumors showed histologically a so-called reticulosarcomatous structure consisting of large and clear cells, but some of them showed a lymphosarcomatous structure consisting of small and dark tumor cells (Fig. 6). Argyrophyl fibres were mostly scantily recognized among tumor cells (Fig. 7).

Metastases in each organ in the latest cases of the diploid type are shown in Table 1. Lymph nodes, lungs, liver, spleen, kidneys and heart were mostly infiltrated with tumor cells macroscopically or histologically. Numerous submiliary tumors were usually found in the omentum and fat tissue of the peritoneal cavity. Tumors in various sizes were always recognized at the insertion portion of the abdominal wall.

Lymph nodes: In most cases retroperitoneal, mesenteric, perigastric and mediastinal lymph nodes were swollen in various sizes corresponding to the duration of the course. Histologically in most cases the affected lymph nodes were occupied by tumor cells, but in some cases only a part of subcapsular sinus of the lymph nodes was infiltrated with tumor cells (Fig. 8). In cases of relatively long duration of the course, other lymph nodes such as the inguinal, axillary or cervical lymph nodes were also infiltrated with tumor cells.

Lungs: Tumor cells were often recognized in capillary vessels. Small vessels or bronchi were often infiltrated with tumor cells, sometimes miliary metastases were recognized with the naked eye.

Liver: Tumor cells were often recognized in sinusoids. In some cases nodular

metastases in various sizes were recognized in the lobule (Fig. 9).

Spleen: Tumor cells were found at first here and there in the red pulp, and then gradually increased in number to occupy the enlarged spleen. Lymph follicles were pressed and disappeared in the extreme cases (Fig. 10).

Kidneys: Metastases were often recognized around the glomeruli or tubules. In some cases miliary tumors were recognized on the surface of the kidney with the naked eye (Fig. 11).

Heart: Small metastases were often recognized in the intermuscular tissue of the heart (Fig. 12).

Bone marrow: Tumor cells were usually found in smear preparations of the bone marrow, but it might be explained to be due to the mixture of the peripheral blood, in which tumor cells were found in most cases. Circumscribed metastasis could not be found in the histological examination.

III. Appearance of tumor cells in the peripheral blood:

Tumor cells appeared in the peripheral blood in the first place 4–5 days after the intraperitoneal transplantation in haemorrhagic types, while they were recognized about one week after the transplantation in tumor types. In almost all cases except those with bacterial infection, tumor cells appeared in the peripheral blood, generally in a few percentages. In some cases tumor cells in the peripheral blood increased in number and the leucocyte number arrived to 100,000 in 1 cmm blood, of which tumor cells occupied about half of the number. Tumor cells which appeared first in the peripheral blood were usually larger and often elongated, but those which appeared in the latest stage were usually round and smaller (Fig. 13). Tumor cells without nucleus were often recognized.

The number of neutrophile leucocytes or monocytes increased usually, accompanied with the increase of the tumor cell number in the peripheral blood. Erythroblasts were often recognized especially in the cases of longer duration.

Various Types of the Hirosaki Sarcoma

The course of the intraperitoneal transplantations and the patho-anatomical findings, which were variable according to various conditions, may be divided into the following 3 types on the whole.

I. Tumor type (First type):

This type is characterized by the marked metastases in lymph nodes chiefly due to the longer duration of the course (about 2 weeks). Postmortem examinations revealed abundant milky ascites in the peritoneal cavity. Lymph nodes in the whole body, especially retroperitoneal, mesenteric, perigastric and mediastinal lymph nodes were swollen markedly in almost all cases. Inguinal or axillary lymph nodes were swollen often moderately, while metastases in cervical lymph nodes were seen in rarely cases. Metastases in lungs, liver, kidneys and heart

were often recognized as numerous miliary nodules. Spleen was often infiltrated with tumor cells, but the intensive swelling was rare. Tumor cells appeared in the peripheral blood first about 7 days after the intraperitoneal transplantation and increased in number gradually, often reaching over 10 % of the leucocytes at the terminal stage.

This type is the most typical one, which might correspond to the human lymphosarcomatosis. The chromosome number of the tumor cells varied, showing a normal curve with the peak of about 37–38 (diploid type).

II. Haemorrhagic type (Second type):

This type is characterized by the haemorrhagic ascites in the earlier stage, which caused subsequently the earlier death of the transplanted rat in about 4–8 days. Postmortem examinations revealed abundant haemorrhagic ascites often accompanied with haemorrhagia in the pleural cavity. Retroperitoneal, mesenteric, perigastric and mediastinal lymph nodes were swollen slightly and the histological examinations revealed usually metastases. Lungs, liver, kidneys and heart often showed the beginning of the metastases. Tumor cells appeared in about 4–5 days after the intraperitoneal transplantation.

The tumor type and haemorrhagic type of the Hirosaki sarcoma appeared alternatively in the successive intraperitoneal transplantations with the interval of several generations. In the cases of bacterial infection the course of the transplantation was prolonged, usually accompanied with vanishment of haemorrhagia, while after the antibiotic treatment the course was shortened, usually accompanied with haemorrhagic ascites. From these facts it may be considered that the tumor cells may be in better condition in the haemorrhagic type than in the tumor type. The violent proliferation of tumor cells in the peritoneal wall, where numerous capillary formations might occur, may be the chief cause of the haemorrhagic ascites.

The chromosome number of the tumor cells of this type also varied, showing a normal curve with the peak of about 37–38 (diploid type).

III. Tetraploid type (Third type):

In one case of the diploid type, tumor cells of tetraploid type increased in number gradually in the longer duration of the course and 52 days after the transplantation almost all the tumor cells were of tetraploid ones. This new type of the Hirosaki sarcoma has been successively transplanted in hybrid rats up to the present time over 130 generations during 2 years.

Tumor cells of this type are markedly larger than those of the diploid type (Fig. 14). The chromosome number varied, showing a normal curve with the peak of about 70–72. Tumor cells of this type show mostly common characteristics with those of the diploid type in other cytological examinations, but the

obvious phagocytizing action upon carbon particles, which was recognized in some larger tumor cells of the tetraploid type, must be emphasized as the peculiar characteristics of this type (Fig. 15).

The course of the intraperitoneal transplantation and the patho-anatomical findings of this type were mostly similar to those of the diploid type. Histological examinations revealed the so-called reticulosarcomatous structure consisting of larger and clearer tumor cells than those of the diploid type (Fig. 16). Tumor cells appeared in the peripheral blood usually in smaller number than in the cases of the diploid type.

On the Mother Cell of the Tumor

The diploid type of the Hirosaki sarcoma should be classified into a type of lymphosarcomatosis chiefly by the following reasons.

1, It originated in the lymph node. 2, Most of the lymph nodes of the transplanted rat were swollen and intensely infiltrated with tumor cells. 3, A few tumor cells appeared usually in the peripheral blood in the terminal stage of the transplantation. 4, The tumor cells showed neither phagocytosis of carbon particles nor peroxydase reaction. 5, The tumor cells resembled cytologically large lymphoblasts in lymph nodes (lymphogonia; Amano) (9).

Histologically the tumor presented often a picture of the so-called reticulosarcoma consisting of large and clear tumor cells, but true reticulosarcomas of the rat, such as the Takeda sarcoma (10, 11) or Usubuchi sarcoma (12, 13) showed obvious phagocytosis of carbon particles. It must be also emphasized that no tumor cell could be recognized in the peripheral blood in these reticulosarcomas.

The Yoshida sarcoma, which is very similar to the diploid type of the Hirosaki sarcoma in cytological findings, must be classified into a type of lymphosarcomatosis.

The tetraploid type of the Hirosaki sarcoma may be considered to be a transitional type to reticulosarcoma from lymphosarcoma by the phagocytizing action upon carbon particles recognized in a small number of cells. Usubuchi and others (14, 15) reported that lymphocytes, monocytes and histiocytes should belong to the same category in postnatal life. According to the authors (7) the alteration of the characteristics of the Hirosaki sarcoma may be explained in the following manner: the lymphosarcomatous type of the Hirosaki sarcoma (diploid type) which corresponded to lymphoblasts in normal cells changed gradually to the transitional type to reticulosarcoma (tetraploid type) which corresponded to monocytes or histiocytes in normal cells.

Mechanism of Metastasis

As described above, postmortem examinations of rats which died of the tumor revealed wide-spread metastases in lymph nodes, lungs, liver, spleen, kidneys and

heart. As tumor cells appeared usually in the peripheral blood in the terminal stage of the transplantation, the mechanism of metastasis in each organ could not be clearly clarified. But even in the cases where many tumor cells appeared in the peripheral blood, several lymph nodes, such as the inguinal, axillary or cervical ones, were often free from metastasis in spite of the wide-spread metastases in other organs. While in the cases where no tumor cells appeared in the peripheral blood, the retroperitoneal, mesenteric, perigastric or mediastinal lymph nodes were usually swollen by metastasis. In such cases the internal organs, such as the lungs, liver, spleen, kidneys and heart were usually free from metastasis.

From these facts it may be considered that the metastases of the tumor in lymph nodes might have occured lymphogenously and that those in other organs might have occured haematogenously. In order to clear up the mechanism of metastasis, following experiments were carried out with the diploid type of the tumor.

I. Intraperitoneal transplantation:

The rats transplanted intraperitoneally with the tumor were killed just before the appearance of tumor cells in the peripheral blood. Histological examinations of the retroperitoneal, mesenteric, perigastric and mediastinal lymph nodes showed

Table 2. Metastases in lymph nodes of rats killed before the appearance of tumor cells in the peripheral blood after the intraperitoneal transplantation. (Diploid type).

Rat No.	Sex	Body Weight (g)	Survival Days	Peripheral Blood	Lymph Nodes									Heart	Lungs	Liver	Spleen	Kidneys	Bone Marrow	Thymus	Pleural Cavity
					Cervical	Axillary (r.)	Axillary (l.)	Inguinal (r.)	Inguinal (l.)	Mediastinal	Perigastric	Retroperitoneal	Mesenteric								
785	♀	70	7	−	−	−	−	−	−	++	+	−	+	−	−	−	−	−	−	−	−
787	♂	65	5	−	−	−	−	−	−	++	+	−	−	−	−	−	−	−	−	−	−
790	♂	100	5	−	−	−	−	−	−	+	+	++	+	−	−	−	−	−	−	−	−
795	♀	70	5	−	−	−	−	−	−	±	−	++	−	−	−	−	−	−	−	−	−
804	♀	90	4	−	−	−	−	−	−	++	++	−	−	−	−	−	−	−	−	−	−
808	♀	70	8	−	−	−	−	±	−	−	+	++	−	−	−	−	−	−	−	−	−
809	♂	100	4	−	−	−	−	−	−	+	++	+	+	−	−	−	−	−	−	−	−
812	♂	100	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
817	♂	140	4	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−
843	♀	80	4	−	−	−	−	±	−	+	+	−	−	−	−	−	−	−	−	−	−
846	♀	70	3	−	−	−	−	−	−	++	++	+	−	−	−	−	−	−	−	−	−
847	♂	70	3	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−

macroscopical or microscopical metastases (Table 2).

A small focus consisting of tumor cells at the subcapsular sinus of these lymph nodes was often recognized as an early indication of metastatic process. The metastatic focus in lymph nodes grew from the subcapsular sinus to the intermediate sinus, and at last the cortical and medullary substances of the lymph nodes were replaced by tumor cells. Except the lymph nodes no obvious metastasis was seen in other organs.

From the experiments it may be said that the metastases in lymph nodes occured lymphogenously and tumor cells in the peripheral blood might have been derived from the metastasis in lymph nodes.

II. Subcutaneous transplantation:

Subcutaneous transplantations of the tumor on the back of rats caused usually formations of large tumor at the site of the transplantations with macroscopical metastases in regional lymph nodes before the appearance of tumor cells in the peripheral blood.

From the experiments of subcutaneous transplantations as well as from those of intraperitoneal transplantations, it may be concluded that the metastasis in lymph nodes occured lymphogenously and tumor cells in the peripheral blood might have been derived from such metastasis in lymph nodes.

III. Haematogenous transplantation:

Haematogenous transplantations of the tumor from the cervical vein carried out on many rats, after which they were killed at various intervals. Histological examinations revealed that the lungs, liver, spleen, kidneys and heart were often infiltrated with tumor cells within several days after the transplantation, while the lymph nodes were mostly unchanged (Table 3).

Several lymph nodes indicated the beginning of metastasis in the subcapsular sinus 6 days after the haematogenous transplantation. The medullary substance and germ centers in the cortical substance of lymph nodes were always free from such metastatic focus. As at the neighbouring tissue of the lymph nodes small haematogenous metastases were often seen at that time, the metastasis in lymph nodes in the haematogenous transplantation may be considered to be lymphogenous as well as in other transplantations (Fig. 8).

Tumor cells appeared in the peripheral blood one day in some cases, 3-5 days in most cases after the haematogenous transplantation. As lymph nodes can not be considered to be the source of the tumor cells in the peripheral blood in such cases, the liver or spleen with their connection to blood vessels must be mentioned.

IV. Conclusion

The sequence of events in the metastatic process of the Hirosaki saacoma (a

Table 3. Metastases in various organs of rats killed at various intervals after the haematogenous transplantation from the cervical vein. (Diploid type).

Rat No.	Sex	Body Weight (g)	Survival Days	Peripheral Blood	Lymph Nodes									Heart	Lungs	Liver	Spleen	Kidneys	Bone Marrow	Peritoneal Cavity	Pleural Cavity
					Cervical	Axillary (r.)	Axillary (l.)	Inguinal (r.)	Inguinal (l.)	Mediastinal	Perigastric	Retroperitoneal	Mesenteric								
3	♂	85	6	+	−	−	−	−	−	##	##	−	±	+	##	+	##	+	±	−	−
4	♀	80	9	##	##	±	−	−	−	##	##	##	##	##	####	##	##	##	+	####	####
5	♂	70	2	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−
11	♀	70	8	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−
12	♂	80	6	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
15	♀	70	10	+	−	+	+	−	−	+	##	##	##	##	±	±	−	##	−	####	####
16	♀	80	2	+	−	−	−	−	−	−	−	−	−	±	±	±	+	−	−	−	−
17	♀	90	4	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	±	−	−
18	♂	85	11	##	##	####	####	−	−	####	####	####	####	####	####	####	####	####	+	####	####
19	♀	90	16	+	####	####	####	−	−	####	####	####	####	####	####	####	##	####	+	####	####
20	♂	120	12	##	##	####	####	−	−	####	####	####	####	####	####	####	####	####	+	####	####
22	♂	60	9	−	##	##	##	−	−	####	+	±	+	####	####	−	−	####	−	####	####
23	♀	55	9	+	−	+	−	−	−	####	+	####	##	####	####	−	−	####	−	####	####
24	♀	70	6	+	−	−	−	−	−	−	+	−	##	+	±	+	−	−	−	−	−
25	♂	75	6	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
26	♀	75	4	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−	−	−
27	♂	75	4	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
28	♂	75	4	+	−	−	−	−	−	−	−	−	−	±	±	+	−	−	±	−	−
29	♂	75	6	+	−	−	−	−	−	+	##	−	−	+	##	##	+	−	±	####	####
30	♂	65	6	+	##	−	+	−	−	##	##	−	+	+	##	+	##	+	±	####	####
31	♀	80	6	+	##	−	−	−	−	####	−	−	−	##	##	+	##	##	+	####	####
32	♀	80	6	+	−	##	−	−	−	####	+	−	−	+	##	±	−	+	±	####	####
33	♂	65	9	+	####	−	−	−	−	####	####	####	####	####	####	##	##	####	+	####	####
34	♀	65	9	+	####	−	−	−	−	####	####	####	####	####	####	##	##	####	±	####	####

lymphosarcomatosis) appears as follows: at first lymphogenous metastasis occurs in lymph nodes, from which haematogenous metastasis occurs in many other organs, such as the lungs, liver, spleen, kidneys and heart.

Summary

1. Cytological and patho-anatomical examinations of the Hirosaki sarcoma revealed that the diploid type of the tumor as well as the Yoshida sarcoma should belong to a type of lymphosarcomatosis and that the tetraploid type should belong to a transitional type to reticulosarcoma.

2. The mechanism of metastasis of the tumor may be considered as follows:

at first lymphogenous metastasis occurs in lymph nodes, from which haematogenous metastasis occurs in other internal organs.

This work was carried out in co-operation with many co-workers in the authors' laboratory.

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Explanation of Plates XXV—XXVII

Plate XXV

Fig. 1. Original rat of the Hirosaki sarcoma showing a thumbball sized tumor in the cervical lymph nodes.

Fig. 2. Section of the original tumor showing tho rest of the structure of the lymph node. H—E. ×100

Fig. 3. Smear of the ascites tumor. (Diploid type). Giemsa's stain. ×1000

Fig. 4. Smear of the ascites tumor showing negative phagocytosis on carbon particles of tumor cells in spite of the marked phagocytosis of macrophage. (Dipoid type). Safranin stain. ×1000

Plate XXVI

Eig. 5. Rat which died of tumor 30 days after the intraperitoneal transplantation showing marked metastases of the axillary and inguinal lymph nodes. (Dipoid type).

Fig. 6. Section of the tumor of lymph nodes showing a lymphosarcomatous structure. (Dipoid type). H—E. ×400

Fig. 7. Silber impregnation of the same specimen as Fig. 6. ×400

Fig. 8. Section of the lymph node showing metastasis in the sinus and primary metastatic focus in the neighbouring tissue on the bottom 6 days after the haematogenous transplantation. (Diploid type). H—E. ×100

Plate XXVII

Fig. 9. Section of the liver showing metastasis in the lobule. (Diploid type). H—E. ×400

Fig. 10. Section of the spleen showing a diffuse proliferation of tumor cells with the rest of the organ on the left. (Diploid type). H—E. ×400

Fig. 11. Section of the kidney showing tumor cells around tubules. (Diploid type). H—E. ×100

Fig. 12. Section of the heart showing tumor cells among muscle fibres (Diploid type). H—E. ×400

Plate XXVIII

Fig. 13. Smear of peripheral blood showing 3 tumor cells. (Diploid type). Giemsa's stain. ×1000

Fig. 14. Smear of the ascites tumor. (Tetraploid type). Giemsa's stain. ×1000

Fig. 15. Smear of the ascites tumor showing positive phagocytoses on carbon particles. (Tetraploid type). Safranin stain. ×1000

Fig. 16. Section of the tumor showing a reticulosarcomatous structure consisting of large and clear tumor cells. (Tetraploid type). H—E. ×400

要　旨

弘前肉腫（白鼠の淋巴肉腫症）

臼淵　勇・安倍弘昌
(弘前大学医学部病理学教室)

1)　弘前肉腫は 1951 年われわれの教室において雑婚白鼠の頸部淋巴節に自然発生した腫瘍を腹腔内に移植し腹水肉腫にかえたものである。

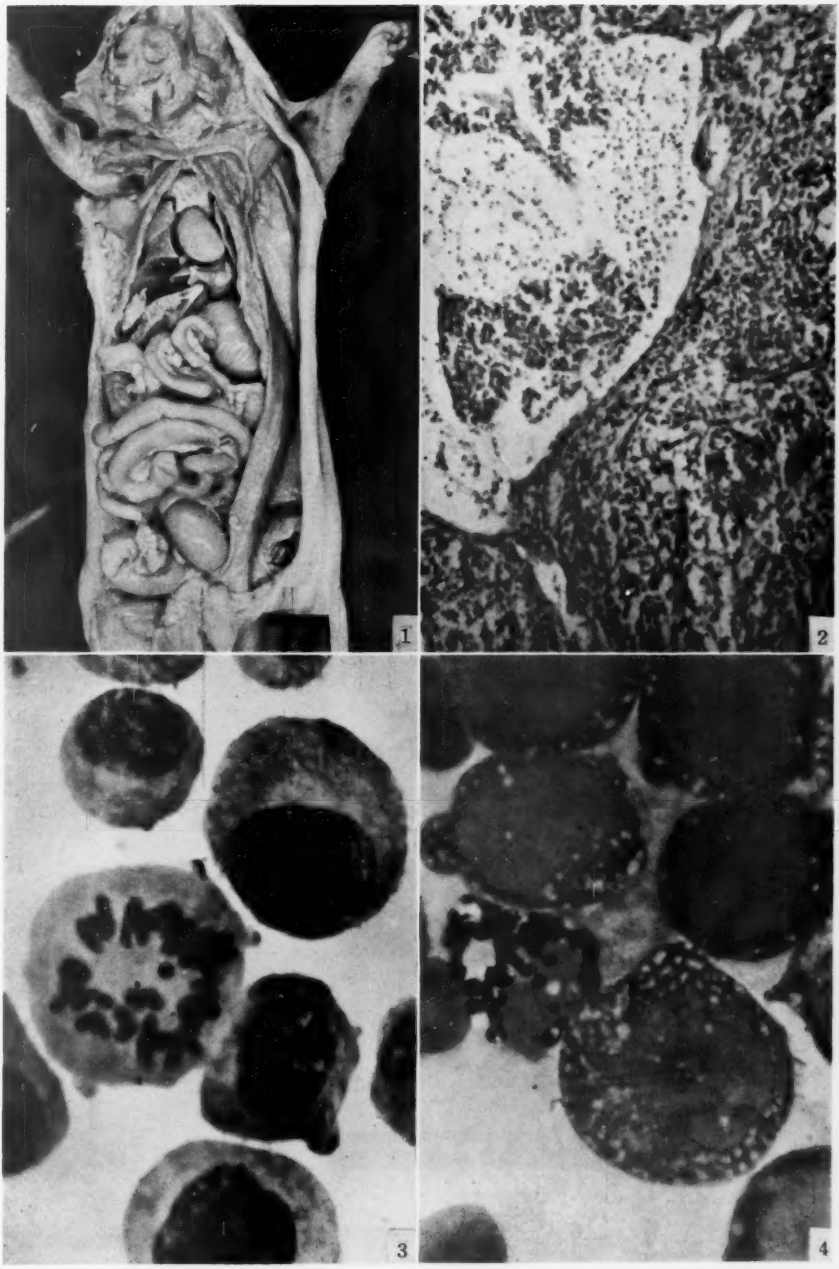
本腫瘍は今日まで 4 年余に及んで主として雑婚，一部 Wistar 系白鼠の腹腔内累代移植が続けられている。

2)　本腫瘍はその経過及び細胞学的所見より diploid 型の腫瘍型 (第 I 型) 及び出血型 (第 II 型) と tetraploid 型 (第 III 型) の 3 型に分けられる。

diploid 型は吉田肉腫に極めて類似する腫瘍であって，われわれは本腫瘍が淋巴節と原発し，淋巴節を系統的に転移し，末期に末梢血に出現すること並びに腫瘍細胞の細胞学的所見 (ペルオキシダーゼ反応陰性，墨粒貪喰能陰性) によって本腫瘍は吉田肉腫とともに淋巴肉腫症に属すべきものと考える。本 diploid 型の腫瘍型は増殖力の比較的弱いときにみられ，出血型は増殖力の比較的強いときにみられるもので本質的の差違は認められない。

本腫瘍の tetraploid 型は一部に墨粒貪喰能陽性のものが認められることによって，淋巴肉腫より細網肉腫への移行型であると考える。

3)　本腫瘍の転移形成は移植局所より所属淋巴節に達し，次第に全身淋巴節に及ぶとともに末梢血に現れて血行性転移を形成するものであることが明らかにされた。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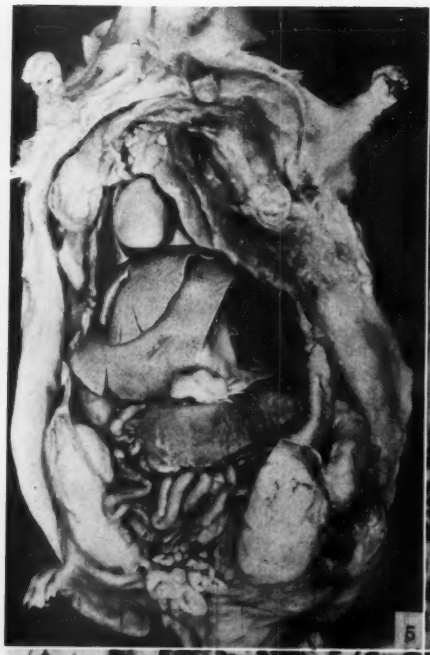
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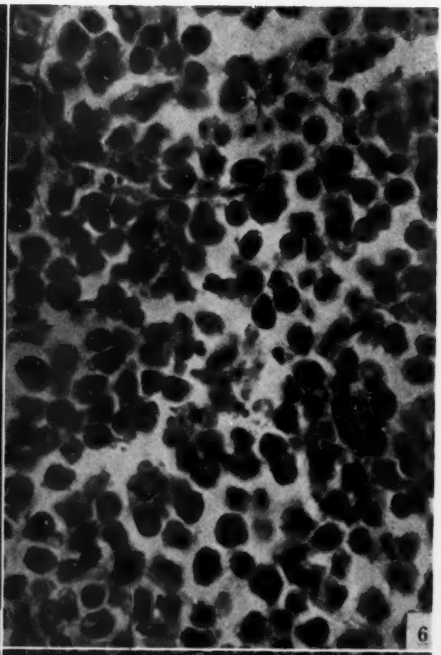
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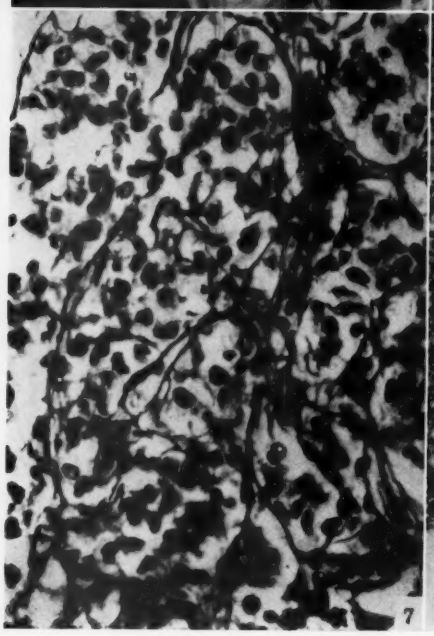
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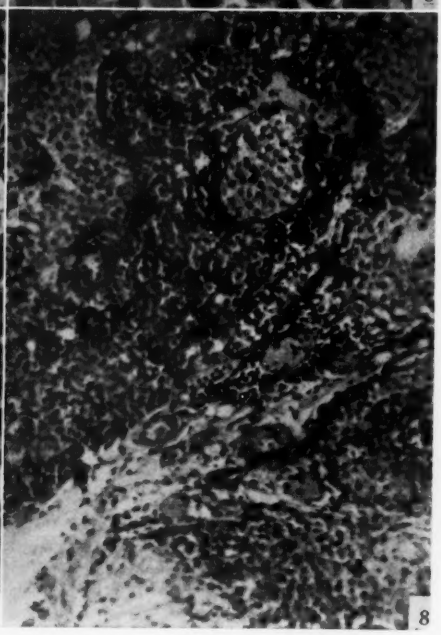
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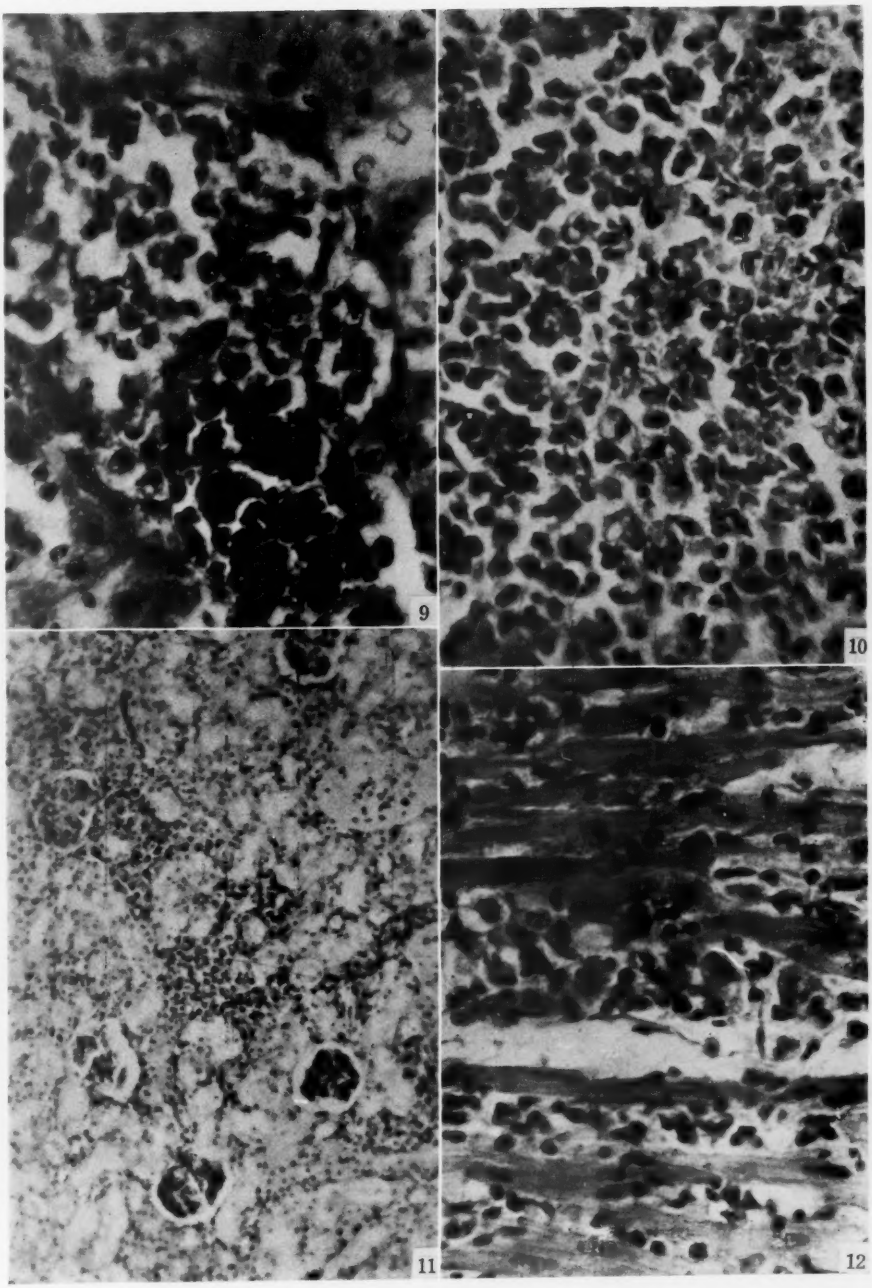
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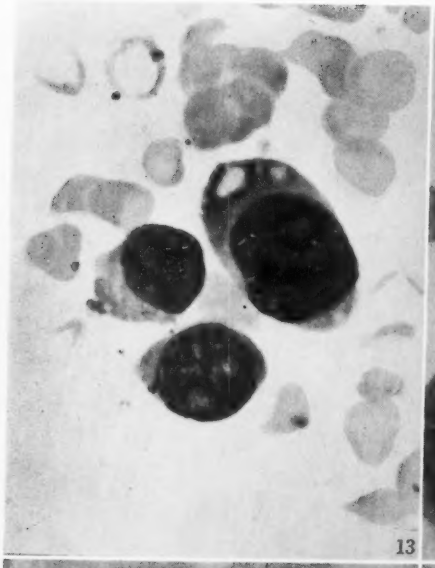
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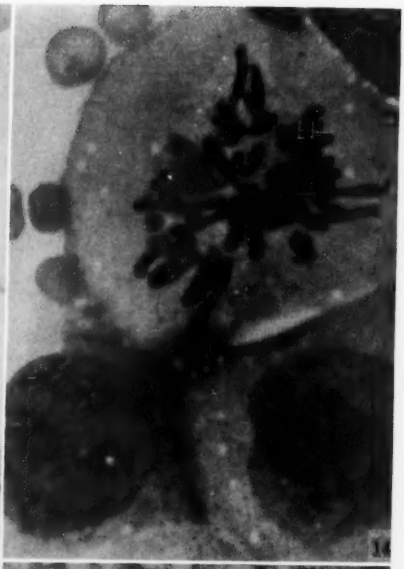
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[GANN, 47, 129~142; June, 1956]

ADENOCARCINOMA OF THE UTERINE CERVIX. A STUDY ON THE HISTOGENESIS (With Plates XXIX—XXXII)

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Practically overwhelming majority of carcinoma of the cervix uteri is interpreted and classified as "epidermoid". According to the opinion of the present authors, fairly large percentages of the "epidermoid" carcinomas of the cervix are of columnar cell origin, as they have demonstrated in their recent paper[15] on the early and in situ carcinomas.

In case their suggestion be correct, definite adenocarcinomas of the endocervix should frequently show such epidermoid transformation. The following data, based on a rather large series of adenocarcinomas of the cervix uteri in the strictest meaning of the term, are presented in order to substantiate this point of view.

Materials and Methods

In toto 92 adenocarcinomas of the cervix uteri comprise the subject of discussion in the present study, among which 32 were hysterectomized, 4 were autopsied and the rest were examined by biopsies, often repeated. All the surgical materials were examined fresh and after fixation in 10% formol, grossly. Five to fifteen blocks were taken from the uterus, tubes, ovaries and parametria. Often serial and step sections were prepared in order to determine the extension and variation of the lesions. Biopsies were submitted in formol and cut in paraffin sections. All sections were stained with H & E, Mallory, PAS and mucicarmin. Detailed comparison of the repeated biopsies and operative specimens were performed.

Definition of Adenocarcinoma

There are considerable variations in the histological appearance of the carcinomas of the cervix uteri, and, as has been pointed out by the present authors, a large series of such material reveals a gradual transition of histologic types, connecting the dual extremities of the typical adencarcinoma and squamous carcinoma. Majority of the cervical cancers appear to have their origin in the glandular or columnar epithelium of the cervix. Therefore, if a term "adenocarcinoma" were used in the histogenic meaning, almost all cervical cancers, both phenomenological adeno-

carcinoma and squamous carcinoma, would be included. In order to avoid misinterpretation and to be sharp in focusing at the point of argument, an extremely clear cut criterion is applied for the adenocarcinoma in this study: there should be in the tumor at least a focus of definite adenomatous structure lined by a single layer of columnar cancerous epithelium with clearly defined margin.

Accordingly, all the other types of lesions even such as commonly accepted as adenocarcinomas are dropped. The omitted group includes such lesions as stratified columnar epithelial carcinomas (epidermoid carcinomas), and infiltrating carcinomas of solitary cell type, even with definite intracytoplasmic mucin droplets. Endometrial carcinomas and lesions with origin in the isthmus were also excluded. Consequently, the group "carcinoma cervicis et corporis" of the WHO-classification was dropped. Metastatic adenocarcinomas and other ill-defined lesions with slightest possibility of metastatic origin (2 cases) are not included.

Result and Discussion

Frequency of adenocarcinoma of the cervix uteri: At the Pathology Department of the Cancer Institute, Tokyo, through a period of 10 years between January 1947 and January 1956, biopsies from 5880 patients were examined. 841 surgical materials of the uterine cervix, all examined histologically, were collected in a period of 6 years from January 1950 to January 1956. All cases operated upon were at least once biopsied. Of the 841 hysterectomized cases 400 had carcinomas of the cervix, of which 32 were adenocarcinomas. Among the 5880 patients examined, 1757 cases had cervical malignancies on biopsy, of which 80 were adenocarcinomas. Additional 18 cases of adenocarcinoma were identified only after detailed histological examination on the surgical materials. Six of the latter represented very early cancers, discussed in the previous study,[3] and were excluded from the following statistical figures. The 92 adenocarcinomas comprised 5.23% of all cervical lesions of neoplastic character. Among the surgical materials they are more frequent (8.0 %).

The frequencies of adencarcinoma among the cervical neoplasms in the literature are listed in Table 1. Hepler, Dockerty and Randall's[6] report was based upon a largest single series of 164 from the Mayo Clinic; Nilsson[12, 13] collected 80. They are omitted because the numbers of the source materials are not stated.

An attention is called to the fact, that adenocarcinoma is more frequent among the operative materials than in the materials which were examined only by biopsy. This is due 1. to selection of cases for surgical treatment because of relatively refractory character of the lesions as compared with the epidermoid carcinomas, and 2. to detection of typical histological features after a thorough search on the surgical materials (in 18 cases in our series).

Data, reported in the literature and based exclusively on the surgical material, coincide almost completely with the present result. Thus, Wheeler & Hertig[15] (1955) found adenocarcinomas in 9.8% among 1,183 cases, and Akazaki[1] (1953) 8.3% among 278 cases.

No significant deviation in the incidence of adenocarcinoma of the cervix among the Japanese population was noticed from that among white races.

Age incidence: Chart 1 shows the age incidence of adenocarcinoma as compared with that of the rest of the cancers of the uterine cervix, representing in the majority "epidermoid" carcinomas. The average age for adenocarcinomas is 46.3 years and shows a shift to a younger bracket than for the more common "epidermoid" type (50.2 years in the Japanese, Masubuchi[3] 1955).

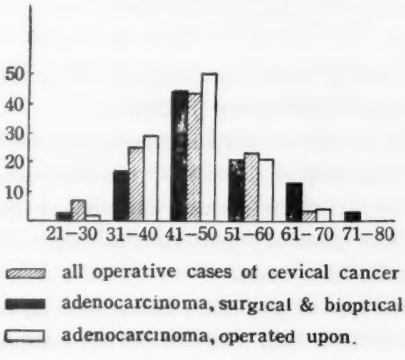
The average age in our series appears to be considerably younger as compared with Hepler et al.'s[6] (ca. 50 years), Limburg's[7] (57.2 years), and Skinner's[16] (51 years). The youngest in our series was 28, whereas the oldest was 73.

Table 1. Frequency of adenocarcinoma of the cervix.[6, 1]

Anthor	Nos. of materials examined	Nos. of Adenocarcinoma	% of all cervical cancers
Bartlett & Smith	560	66	11.7
Limburg	3057	65	2.1
Baldwin	722	55	7.6
Bowing & Fricke	1491	53	3.5
Chambers	678	50	7.3
Akazaki	278	23	8.3
Tanaka & Oota	1757	92	5.23

Chart 1.

Age incidence of adenocarcinomas of the cervix uteri, as compared with that of all cervical malignancies.

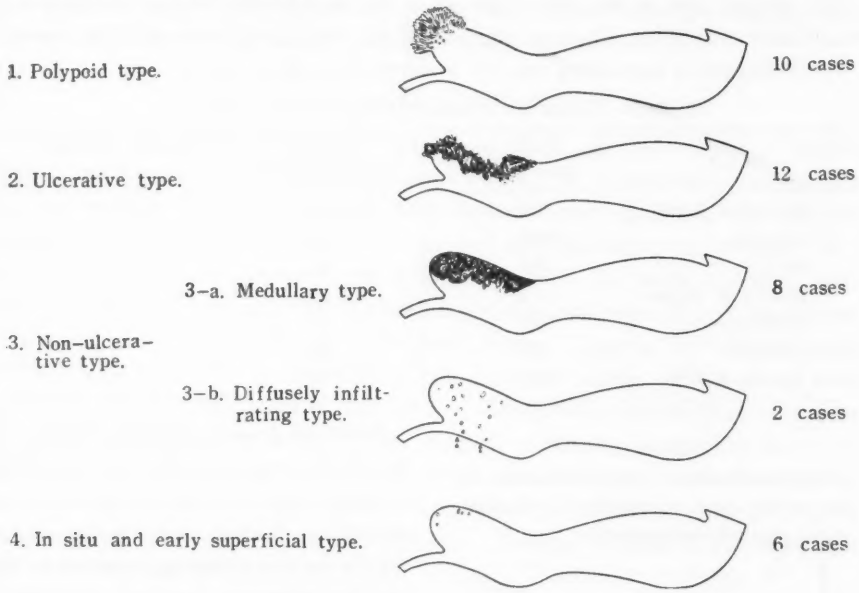


Mode of growth and extension: In all of the surgical materials the endocervical mucosa was the principal site of lesion. Sixteen showed the center of the lesions situating adjacent to the squamo-columnar junction, and seven located near the isthmus. In only one (G-719), the tumor had arisen from deepseated endometriosis and secondarily invaded the endocervix. Six originated from the depth of the endocervix and just reached the surface, while the bulk of the tumors infiltrated the myometrium. Direct extension to the neighboring uterine segments was fairly

extensive in some cases. Almost complete destruction of the portio was seen in nine cases. Five cases showed ascending involvement of the corporal endometrium.

It should be emphasized that an adenocarcinoma can not infrequently originate from the portio uteri. Fourteen were grossly carcinomas of the portio and five of which were exclusively located in the area. This would be readily understood when one surveys a large series of uterine material and comes to know the fact that, in many pathological conditions the endocervical mucosa can extend to the previous squamous epithelial areas.

Chart 2. A diagram showing macroscopical growth-patterns of the adenocarcinomas of the cervix uteri. The figures on the right represent number of cases encountered among the surgical materials.



The growth of adenocarcinomas of the cervix uteri was classified into a few types in the gross pattern, which are diagramatically shown in Chart 2.

1. Polypoid type: This type shows califlower-like or polypoid exophytic growth protruding from the external os (Fig. 1). The largest (G-631) of ten examples classified in this group reached 7.6×6.3×3.6 cm in the greatest dimensions and entirely filled out the vaginal vault. Grossly papillary pattern with signs of secretion makes macroscopic diagnosis easy in these cases.

2. Ulcerative type: This is an endophytic type of medullary tumor forming an ulcer at the external os or of the endocervical wall. Twelve of our present

series belonged to this group. The lesions recall the common disk-like carcinomas of the gastrointestinal tract. The cervix uteri is usually not hypertrophied. As such lesions are often invisible at casual examination of the portio, careful endocervical biopsies are prerequsit for correct diagnosis.

3. Non-ulcerative type: Ten cases belonged to this group. Although the cancers were almost exclusively endophytic, the endocervical linings were smooth and no ulcerative process was observed.

a. Medullary subtype: Eight of our cases were medullary and had very distinct limitation. Histologically they were well differentiated and showed organoid pattern. No necrosis was seen.

b. Diffusely infiltrating subtype: In two cases, which belonged to this subtype, the cancers infiltrated deep into the fibromuscular layers without sharp boundary. There was no nodular mass, so that the cervices appeared with close resemblance to myomatous hypertrophy (Fig. 2). Histologically, they were either adenomatous (Fig. 12) or scirrhous, and represented the two cases among the whole series in which the parametria were reached. Considerable difficulty in clinical diagnois is expected.

4. In situ and early superficial type: This specific type will be discussed separately and in detail elsewhere. Six such cases were excluded from the overall statistics in this series.

Limburg[7] classified the adenocarcinomas of the cervix uteri into three types, exophytic, endophytic and crater-forming types. Nilsson[12,13] followed Limburg's example in the principle, but considering the localization of the lesions, divided them into seven groups. Norris'[14] four groups were ulcerative, papillary, nodular and diffuse types.

Metastasis: Among the radically hysterectomized thirty cases, six showed metastasis to the external iliac and/or obturatory lymph-nodes. This comprised 20 % of all adenocarcinomas of the cervix in which a systemic examination of the regional nodes was performed, and coincides with the data (20 %) of Nilsson[13] (1935). There was no difference in the rate of metastasis between adenocarcinoma and epidermoid carcinoma of the cervix. From our Institute, Masubuchi and Yanagihara[9] (1954) reported a metastatic rate of 18.1 % for the overall cases (154 cases) of cervical cancers of the uterus.

There has been no description concerning histological features of the metastatic foci in cases of adenocarcinoma of the cervix uteri. In our series, one case (G-631) showed a pure tubular adenocarcinomatous structure, and another (G-763) a purely epidermoid pattern in the metastatic foci, whereas the other four represented a fair mixture of the adenocarcinomatous and epidermoid types. In all six cases the primary lesions were composed of mixed histology as will be discussed later.

Histocytological Findings

Histological classification: Survey of the collected materials revealed the presence of some fundamental histologic types in the endocervical adenocarcinomas. Some showed well differentiated papillary structures (Fig. 1 & 19), and the others tubular formation often of duct-like (Fig. 12 & 15) or acinar appearance (Fig. 7). As will be discussed elsewhere, six of our cases were confined within the preformed architectures of the endocervical glands and were consistent with adenocarcinoma in situ.

a. Papillary type 58 cases (63.1 %)
b. Tubular duct-like type 21 cases (22.8 %)
c. Acinar microcystic type 13 cases (14.1 %)
d. In situ type 6 cases+

About one-third (30 cases) are homogeneously simple in histological pattern all over, but others, comprising the majority of our materials, contain areas of solid cell masses in variegated extent, as it is shown in Table 2. This mixture of histological patterns in a definite adenocarcinomas of the cervix uteri is the most important point in understanding the histogenesis of carcinoma in general, appearing in this particular location, and will be fully discussed below.

Attention should be paid to the fact, however, that, apart from the variation of histological pattern in a single entity of carcinoma, there are also such cases in which two independent primary carcinomas, one adenocarcinoma and the other squamous carcinoma, have developed in two adjacent areas and have come into contact or close together. Findings, consistent with this separate primaries, were encountered twice, in G-270 and R-19353, during this survey.

Sometimes, although a biopsy showed fairly simple adenocarcinomatous pattern,

Table 2. Structure of adenocarcinomas.

Pattern / Differentiation	Hemogenously simple structure	Mixed & solid area, extent			Total
		+	++	+++	
Papillary	23	12	8	15	58
Gland like pattern, mainly tubular	7	5	1	8	21
Mainly acinar	0	1	2	10	13
Total	30	18	11	33	92
		62			

hysterectomized material offered high complexity of histological types. Eighteen of our present cases were re-classified into the mixed group after thorough examination of the material obtained at operation.

Cytological patterns and differentiation: The cancerous epithelia maintain in the majority resemblance with certain epithelial types of the Muellerian duct origin.

a. Thus, 29 of the cases has mucus secreting clear cell epithelia which resemble with those of normal endocervical glands (Figg. 3 & 12);

b. Some (8) had cancerous epithelia with the outlook of the dark isthmus epithelium (Figg. 5 & 6);

c. 51 had epithelia of dark-staining cuboid cytoplasmic character and with fine-droplets of serous secretion, not unlike those of the corporal endometrial gland (Figg. 7, 8, 9, 10, 13, 15 & 16);

d. In a few cases (4) they had much similarity to the tubal epithelium with those dual cellular types (Fig. 11); and

e. Two adenocarcinomas were so anaplastic that no physiological counterpart of the epithelium was found.

Thus, in the majority of the lesions, the tumor cells had fairly distinct features as to suggest their possible origination, except in Group e.

Any of the above cellular types could be found in association with the solid histological pattern, but Group c appeared to be most liable to solidification. We would like to point out the close similarity of the cellular component of this group with the basal cells commonly encountered in the endocervical pathology and designated as basal cell hyperplasia. The cells of this type are multipotent: they can not only differentiate both into the typical endocervical gland and into the isthmus type, but also can continue to heap up into a solid mass, which secondarily differentiates in an epidermoid pattern, just like in the epidermization of the endocervical epithelium of healing erosin.

The Epidermoid Patterns in Adenocarcinomas

Although, all of the present materials fullfilled the requirement of the criteria of adenocarcinoma set at the beginning of the study, in about one-third (33 cases) solid cellular nests occupied substantial portions of the tumor masses (Table 2). More or less conspicuous solid pattern was seen in the other 29 cases. One can get an impression of epidermoid carcinoma from such areas so easily (Figg. 9 & 18) that among them eighteen were designated at first (mainly on biopsy) as epidermoid. Thorough examination revealed definite adenocarcinomatous characteristics in the other parts of the same mass.

As similar observation has been done frequently by the present authors also in the neoplastic changes of such other organs as lung[17,10] and upper respiratory

tract, this point is believed to deserve full description and discussion.

The solid areas showed variegated patterns of the cancerous epithelium concerned.

a. Often (in 39), the epithelium piled up into solid nests and had close resemblance with that seen in the epidermization in non-neoplastic cervix. The epithelium, originally cylindric in nature, showed stratification intermingled with a few mucin-secreting cells. Thus the solid nests had irregularly distributed large and small mucicarminophilic globules (Figg. 3, 4 & 8), although no definite lumen was formed in the particular areas. Such solid areas were frequently encountered in the well differentiated clear cell type of endocervical adenocarcinomas.

b. Among our series there was not a case of adenocarcinoma showing the typical feature of adenoacanthoma most commonly observed in the endometrial malignancy. Actually, one such case, at first thought to be endocervical primary, showed unexpectedly diffuse involvement of the endometrium. It was interpreted as having a corporal-endometrial origin and excluded from the present series. (As will be discussed later, some endocervical cancers can originate on the basis of endocervical endometriosis).

Frequently, closer resemblance with squamous cancer was observed, when the principal cytologic pattern of the cancer belonged to the basal cell type (Figg. 9 & 10). Our series contained 19 of such examples. In six cases superficial extension of the cancer replaced the surface lining epithelium and even the squamous one of the portio, simulating locally the in situ epidermoid carcinoma (Fig. 14). None or only trace of mucicarmin-positive mass was found in such areas. Small pearls were rarely seen (in 3) (Figg. 15 & 16), although true spinous prekeratinizning layers were not observed.

c. Most often (in 19), the solid masses chiefly represented ill-defined cylindropolygonocellular proliferation without sign of keratinization (Fig. 17 & 18). Sometimes small intracellular mucicarminophilic droplets were seen in such areas. When large portions of the carcinoma undergo such transformation, it offers the most common features of the uterine cervical carcinomas, popular with the designation of "epidermoid" of many authors.

d. Solid nests of very anaplastic cells were seen in only four cases.

Transition between the glandular and solid areas may be very gradual in one tumor and adrupt in the others. In some cases a very complicated intermingling of the two patterns was observed. Usually it was impossible to demonstrate such a clear transitory area, with the dual extremities at the same time within the scope of a microscopic field. Naturally, a remnant of normal endocervical gland in the solid infiltration of carcinoma may simulate such glandular pattern of neoplastic process and should be carefully excluded.

Table 3. Grading of cellular anaplasia.

Pattern / Broders	Hemogenously simple structure	Mixed & solid area, extent			Total
		+	++	+++	
1	G-71(S. J.), 695, 721, 724 R-1134, 15980, 19330, 19353, 19924 T-6118	G-598, (S.T.), 631, 823			13
2	G-386, 472, 645, 951 R-36, 189, 264, 3506, 3619, 4337, 4859, 5573, 5698, 6106, 8007, 11133, 13640, 18141, 18689, 19381	G-78, 301, 304, 418 R-2440, 3964, 4116, 14121, 19190	G-270, 711 R-1037, 3025 (S-63), 4449	R-666, 699, 719, 763, 765, 780, 804 R-56, 2449, 3416, 3513, 4900, 7911, 15538, 16585, 18411 S-200 T-6898, 8249	53
3		G-282 R-2907, 4633, 8007, 15950, 18141	G-4929, 5564, 9591, 13844, 15497, 19775	G-201, 283, 305, 803, 805, 854 R-6979, 7238, 8213, 9515	22
4				G-207 R-4850, 5846, 6752	4
Total	30	18	11	33	92
		62			

Many cases, which were thought to be double cancers at the first sight, i. e. independent glandular and epidermoid carcinomas in one cervix, revealed after a careful study of the whole specimens that they were actually single tumors showing the dual pattern in cellular differentiation.

Grading

Grading of cellular anaplasia after Broders was tried in the present series. Most of the papillary type cancers fell into the less anaplastic group. Incidence of a solid cell mass appeared no to go paralled with the anaplasia-grading (Table 3). This suggests an intrinsic potentiality of the endocervical epithelium to differentiate into an epidermoid pattern, regardless to anaplasia.

The presumption, that formation of solid cellular nests in any adenocarcinoma means higher grade of anaplasia, is apparently false. Epidermoid pattern in an adenocarcinoma of the cervix uteri means only a difference in direction within the scope of physiologic potentiality of the original epithelium.

Adenocarcinoma of Special Origin

A. **Origin in the adenomyoma:** A polypous growth of extreme size in one of our series revealed fibromyomatous stroma in the core, which fact might most suitably be interpreted as malignant transformation of an adenomyomatous polyp (Figg. 19, 20 & 21). The cancer cells in this case belonged to the clear cell type and showed in part simulation of epidermization.

B. **Origin in the endometriosis:** One of the cases (case-G-719) showed definite evidence of endometriosis of the cndometriosis of the cervix (Figg. 22 & 23). Adenocarcinoma encountered in the case appeared to have originated from the deeper myometrial layer near the portio, where irregular cystic structures of non-malignant epithelial lining, accompanied by a little amount of cytogenic stroma, were seen embedded in the posterior lip. The cancer belonged to the basal cell type, regarded rather uncommon in the endometrial carcinoma.

C. **Gartner's duct carcinoma** was not encountered in our series.

D. **Stump carcinoma:** Five of our cases (5.88%) were adenocarcinoma arising from the stumps after supravaginal hysterectomy, invariably because of myomatous uterus. They occurred 1.5 (R-2907), 1.5 (G-711), 2.5 (R-1037), 3 (R-16583), and 5 (R-3619) years respectively after amputation. Origination of the cancers from other than the endocervix was most unlikely. One was simple glandular type, and the other four contained various amount of solid cell nests.

The five adenocarcinomas in our series constitute as much as 19% of all primary stump carcinomas (26 cases) observed during the period of this survey. The rest belonged to the squamous variety. There seems to be a remarkable discrepancy in the rate of incidence of adenocarcinoma at the stump of cervix uteri in the Japanese and American female population. The comparable data of Dodds and Latour[2] show that adenocarcinomas comprise only 5.3% of their 75 stump cancers, whereas 9.1% of their 44 adenocarcinomas of the cervix originated from the stump of supravaginal hysterectomy.

Adenocarcinoma associated with cervical tuberculosis: Association of tuberculosis, all with densely disseminated miliary tubercles, was seen in four of our series(G-711, R-9515, G-631, G-71). On one occasion (G-71), because both clinical and bioptical pictures were almost completely masked by the tuberculous processes, a wrong diagnosis was reached. Autopsy revealed extensive colloid carcinoma of the pelvic cavity with fairly well preserved bilateral tubo-ovarian structures and, peculiarly, without definite sign of preexsistent tuberculous lesion except fibrous adhesions. Case G-711 had an adenocarcinoma of the cervical stump concomitant with tuberculosis (Fig. 24).

Reports on the relationship between tuberculosis and carcinoma of the female genitalia are scarce. Hata[5] (1947) reported one case of concomitant squamous carcinoma and tuberculosis of the cervix uteri. Moricard[11] (1954) appears to be

the only foreign author to report one similar case. There has been no reference in the discussion of tuberculosis and adenocarcinoma of the cervix.

In our adenocarcinoma series complications of cervical tuberculosis was as frequent as 4.4 %. During the same period of study, two additional cases of tuberculosis associated with epidermoid carcinoma of the cervix were observed. Relatively frequent association of these two conditions (6 cases among total 1,757 cervical malignancies) in our material may be related to the high incidence of the two diseases in this country. According to Masubuchi,[8] 0.1 % of all patients seen at the Gynecology Clinic, Cancer Hospital, Tokyo, had cervical tuberculosis and 0.2% endometrial tuberculosis.

It is also of note that two-thirds of the cervical cancers complicated with tuberculosis belonged to the glandular type. Tuberculosis and other inflammatory stimulation might have something to do with local cancerization.

Conclusion

1. Definite adenocarcinomas comprised about 5.23% of all (1757) uterine cervical malignancies.

2. They can originate from the cylindric epithelium of the normal and polypous endocervical mucosa, on the basis of cervical endometriosis and from Gartner's duct. The last example was not found in this series.

3. Some of the adenocarcinomas have hemogenously simple glandular features throughout, but others can very often undergo transformation into solid pattern, consistent with "epidermoid" carcinomas.

4. Detailed examination on hysterectomized materials sometimes reveals originally adenocarcinomatous nature of casually observed solid carcinomas, often designated as epidermoid.

5. From our present series many cervical cancers, which, at least in parts, clearly indicated their glandular origin, were omitted. Thus adenocarcinomas of wider sense of the meaning with traceable signs of aborigin from the columnar elements of the cervix, should be much higher in percentage incidence among the entire group of cervical malignancies.

6. The present data, together with the facts advanced in the previous study on the topographic distribution of early carcinomas, apparently indicate the importance of the columnar epithelial elements of the endocervix in the histogenesis of the cancers of the uterine cervix in general.

7. Many of the solid and partly epidermoid carcinomas are direct derivatives of the columnar epithelium. It appears unnecessary to presuppose squamous metaplasia of the columnar epithelium in the common histogenesis of the cervical carcinoma.

8. There are definitely very early adenocarcinomas of the endocervix which can manifest themselves as in situ epidermoid lesions at the front of their superficial spread. This appears to have been missed by previous investigators.

9. There were five (5.88 %) adenocarcinomas occurring at the stumps. They comprised 19% of all stump cancers seen in the same period.

10. Adenocarcinomas of the endocervix can be associated with tuberculosis. This remarkable condition was discussed on the basis of 4 cases of personal experience.

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Legends for the Photographs

Fig. 1. Adenocarcinoma of the cervix uteri (G-721), showing polypoid-papillary gross pattern. The tumor epithelium belongs to the clear, well-differentiated cell type. Higher magnification reveals definite invasion of the stroma.

Fig. 2. Non-ulcerative, diffusely infiltrating type of adenocorcinoma of the cervix (G-695). The cervix is enlarged keeping a good overall proportion. The wound at the far left indicates the site of an endocervical biopsy.

Fig. 3. A typical adenocarcinoma of the cervix (G-304), showing the characteristic cell components which recall normal endocervical epithelium at the left. Transition to the "epidermoid" pattern is well demonstrated on the right hand.

Fig. 4. A little more disorderly pattern of a clear cell type adenocarcinoma of the cervix (R-3513); resemblance with early epidermoidalization.

Fig. 5. Papillotubular pattern in an adenocarcinoma of the cervix (G-270), showing close resemblance with normal isthmus endometrium.

Fig. 6. Another isthmus endometrium type of adenocarcinoma of the cervix (R-19353), demonstrating transition to solid cell nests at far right.

Fig. 7. The acinar microcystic pattern in an adenocarcinoma of the cervix (G-854), consisting of granulated cuboid epithelium. Piling up of the cuboid basal cells simulates the "epidermoid" pattern.

Fig. 8. A tubular type medullary adenocarcinoma of the cervix (G-666) with more advanced, well developed solid alveolar structures.

Fig. 9. An adenocarcinoma of the cervix uteri (G-780), consisting of dark cells recalling basal cell proliferation. The left field view is almost consistent with the common "epidermoid" carcinoma but definite glandular tubular structures are shown on the right hand.

Fig. 10. Another solid alveolar pattern of adenocarcinoma of the cervix (G-305), in which the constituents retain the original cylindric cell character.

Fig. 11. An example of the peculiar tubal epithelial type of adenocarcinoma of the cervix (G-386). The cancer epithelium reduplicates the dual cell type of the Fallopian tube.

Fig. 12. Diffusely infiltrating duct-like structure of the non-ulcerative type of adenocarcinoma of the cervix (G-695). In this and another case the parametria were involved. See also Fig. 2.

Fig. 13. An example of adenocarcinoma of the cervix (R-2449) showing the transition of the dark staining cancer cell nests from glandular to epidermoid pattern. Some areas show "spinous" differentiation of tumor cells, although mucin droplets are scattered throughout.

Fig. 14. Interstitial and intraglandular extension of an adenocarcinoma of the cervix (G-805), showing almost equivalent morphology with that seen in the common epidermoid carcinomas.

Fig. 15. Occasional epidermoid differentiation in a more anaplastic adenocarcinoma of the cervix (R-4929).

Fig. 16. More prominent squamous metaplasia occurring in an adenocarcinoma of the cervix (R-14121). Some pearls are also shown.

Fig. 17. Another example of epidermoid metaplasia in adenocarcinoma of the cervix (G-854).

Fig. 18. The major portions of the same carcinoma (G-854), shown in Fig. 17, almost completely reduplicate the common epidermoid carcinoma of the cervix.

Fig. 19. A well-differentiated cystopapillary pattern in a large polypoid adenocarcinoma of the cervix (G-631). A part of benign adenomatous endocervical glands at the lower left hand.

Fig. 20. A higher power magnification of the boundary between the benign and malignant adenomatous structures in the case G-631, shown in Fig. 19.

Fig. 21. In some areas, the cancer (G-631), shown in the Figg. 19 & 20, forms solid cell nests, simulating closely an epidermoid carcinoma of the cervix.

Fig. 22. An adenocarcinoma has arisen from the cervical endometriosis in the right lower field (G-719). The glandular and cytogenic stromal components are clearly demonstrated in the other parts of the picture.

Fig. 23. Abortive epidermoid metaplasia in the adenocarcinoma (G-719), shown in Fig. 22. It is not consistent with adenoacanthoma of the endometrium.

Fig. 24. An adenocarcinoma of the cervix uteri associated with tuberculosis (G-711). The cancerous epithelium is the common dark cell type. A tubercle at the lower right hand.

要　旨

子宮頸部腺癌：　組織発生学的研究

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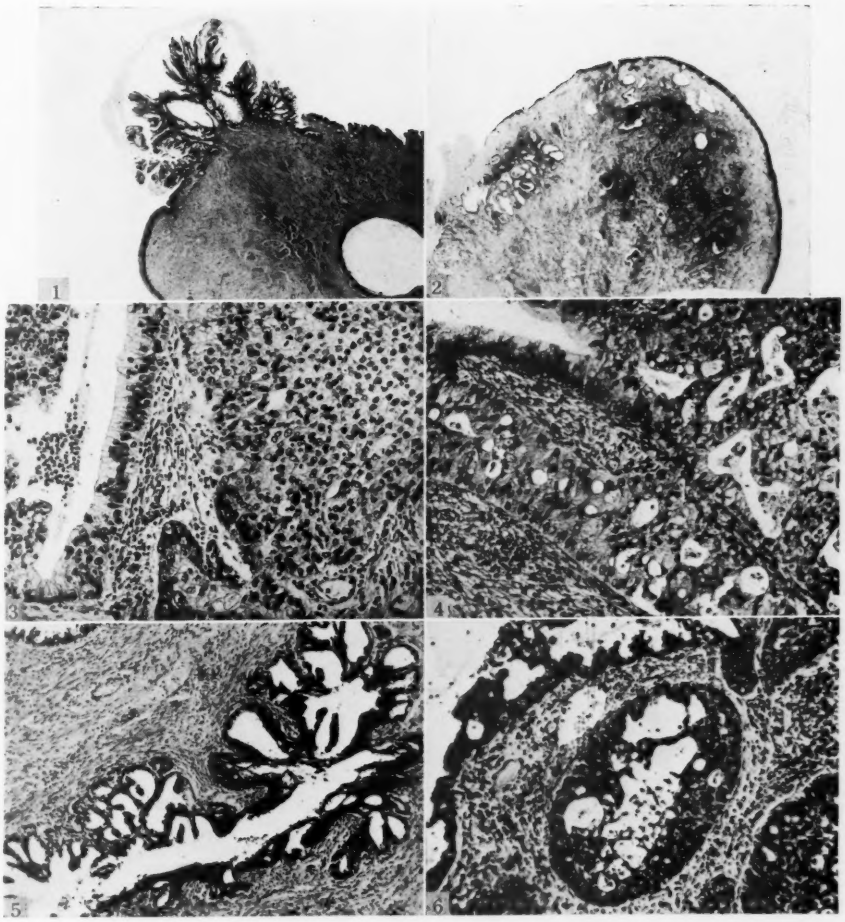
最近約 10 ケ年間に癌研究所病理部で扱った子宮頸癌 1,757 例の組織学的検索により 92 例 (5.23%) の腺癌及び文献上極めて稀有な上皮内及び早期腺癌 6 例を得た。

これらの内，約 $^1/_3$ は単一型であるが残余は充実巣ないし層状配列部を合併せる混合型である。混合部の多くは，非癌時の類表皮化生と同様の過程を経て，子宮頸癌の大部分を占める扁平上皮型癌の像を呈する。著者らは先に上皮内癌及び早期癌の研究において，従来の子宮頸癌の発生母地は扁平上皮域が主であるとの説に反して円柱上皮起源が圧倒的に多いことを証明したが，今回の研究においても同様の結論に到達した。

また，線維腺腫性息肉より発生した巨大腺癌，頸管部内膜症より発生した頸部腺癌の特殊起源の各 1 例，断端癌としての子宮頸部腺癌 5 例，同一子宮頸部に結核を合併せる腺癌 4 例は何れも文献上極めて稀有である。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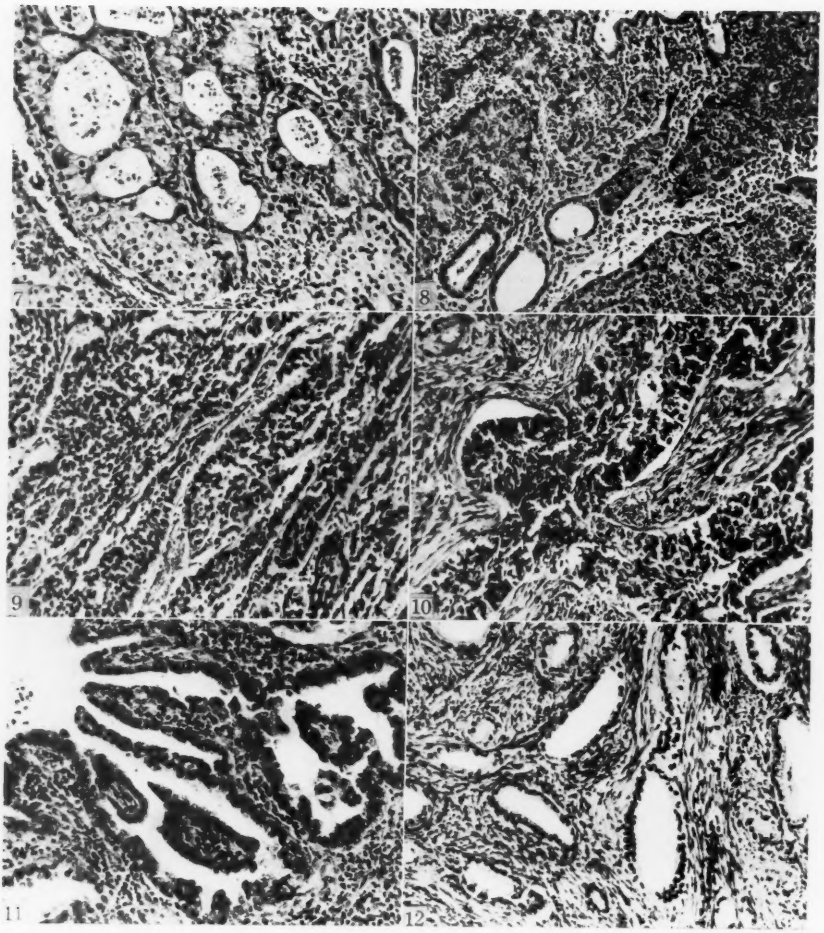
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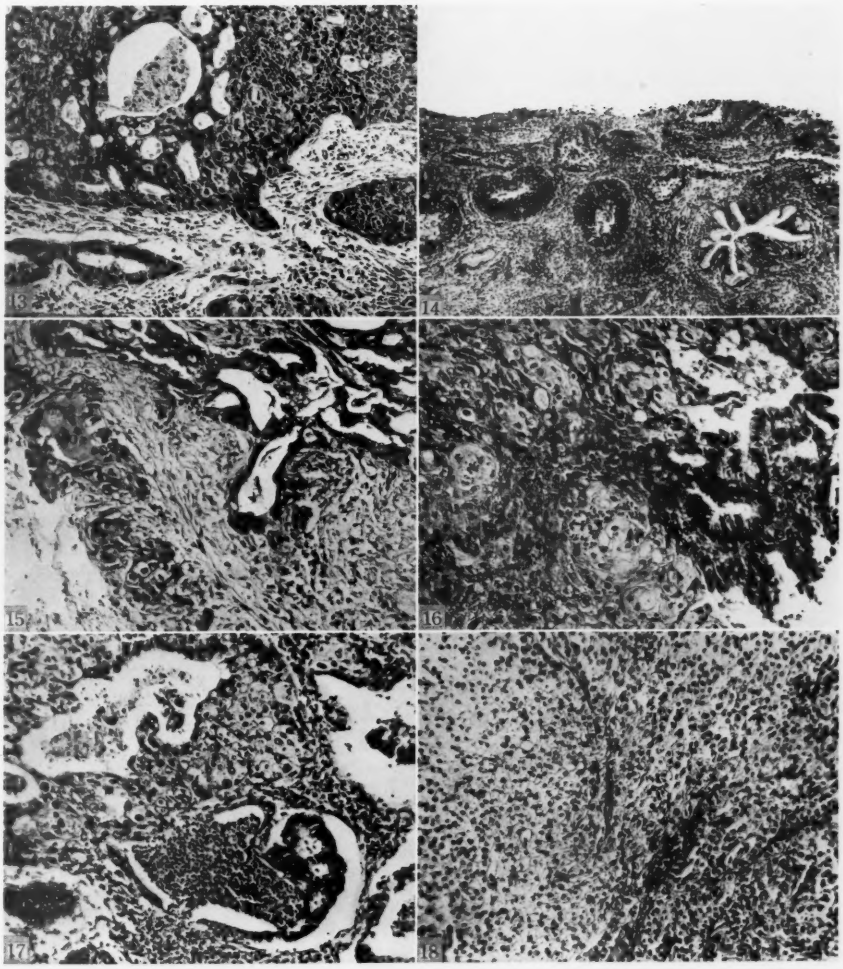
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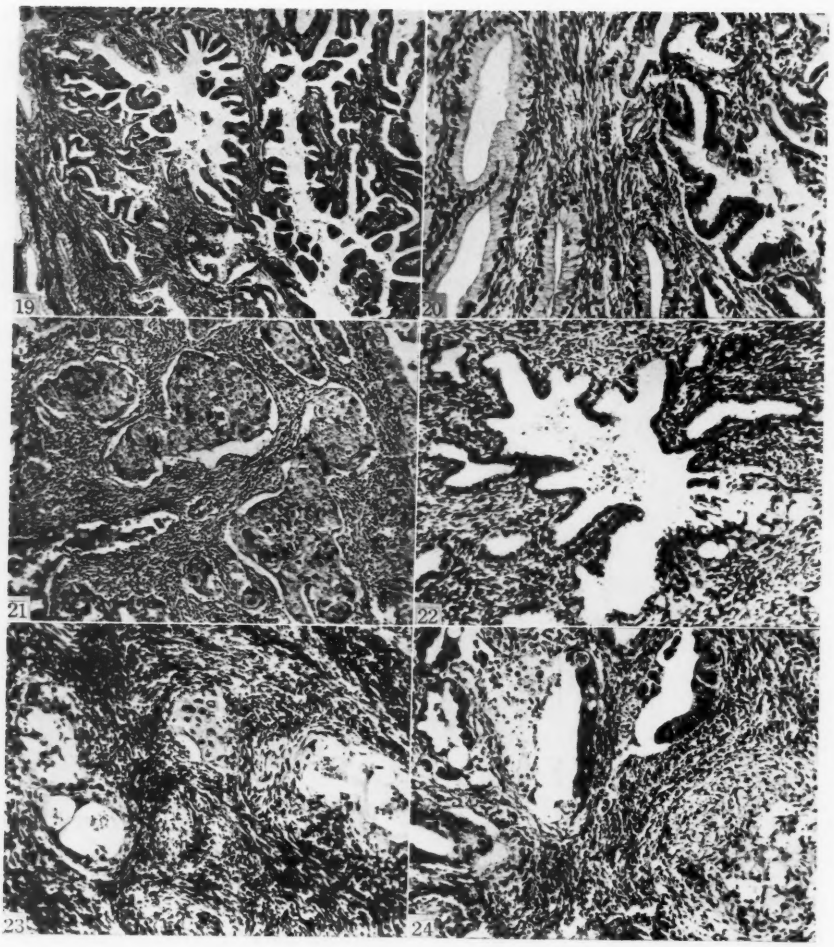
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[GANN, 47, 143~152; June, 1956]

GASTRIC NEOPLASMS OTHER THAN CARCINOMAS: A HISTOLOGICAL AND STATISTICAL STUDY ON 1489 RESECTED STOMACHS (With Plates XXXIII~XXXVI)

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Among the Japanese population carcinoma of the stomach occupies the highest frequency rate in all neoplastic diseases. Recent statistics (Segi) revealed that 60% of all cancers among Japanese males were gastric cancers. They are also most frequent in female population (40%). Thus gastric surgery is one of the greatest concerns in the treatment of malignant growths in Japan. But, unfortunately a report on a large consecutive series of the surgical materials from a single institution with reliable pathologic examination has been lacking in this country. The Pathology Department of the Cancer Institute, Tokyo, has been active since 1934, but the data before the last World War were destroyed. The present report is based upon the study on a consective series starting in September 1947. Some statistical data concerning the histological classification of the gastric carcinomas has been presented previously[13] (1952).

Tumors of the stomach other than carcinomas are rather rare. Borrmann[1] found only five sarcomas of the stomach among 11,475 autopsies which contained 240 carcinomas. Such rarer tumor types as paraganglioma and chorionepithelioma have been found in the world literature, but they seem to be of no practical importance.

Materials and Method

The materials consist of 1489 resected stomachs examined at the Pathology Department of the Cancer Institute, Tokyo, between September, 1947, and November, 1955. All materials were examined fresh and after formalin fixation. Every lesion and representative parts of the stomach were studied on many paraffin sections.

Relative incidence A total of 50 (3.3%) non-carcinomatous gastric neoplasms were found among this consecutive series. Most common conditions encountered during the same period are carcinomas 996 (66.9 %) and chronic ulcers 343 (23%), which are also listed for comparison (Table 1).

Table 1. List of neoplasms in gastrectomized materials, Sep. 1947—Nov. 1955.
(Path. Dept., Cancer Institute, Tokyo, 1956)

	Number of cases	%in the entire materials	% in neoplastic lesions	% in the subgroup
Total gastrectomized materials	1489	100.0		
1. Non-neopastic lesion:	443	29.8		
Gastric ulcer	343	23.0		
Duodenal ulcer	90	6.0		
Gastritis	10	0.67		
2. Neoplastic lesion:	1046	70.2	100.0	
A. Epithelial neoplasma	1024	68.8	97.9	
Carcinoma	996	66.9	95.2	
Epithelial polyp	28	1.9	2.7	
benign polyp	23	1.5	2.2	
malignant polyp*	5	0.3	0.5	
B. Non-epithelial neoplasma:	22	1.5	2.1	
Myogenic origin:	12	0.81	1.2	
benign leiomyoma	8	0.54	0.76	36.4
leiomyosarcoma	4	0.27	0.38	18.2
Lymphoblastic origin:	9	0.60	0.86	
small-cell lymphosarcoma	1			
reticulum cell sarcoma	8	0.54	0.76	36.4
Neurogenic origin	0			
Miscellaneous	1			
malignant melanoma**	1			

* Autochthonous malignant transformation of originally benign polypi.

** There was no other primary focus identified.

A. Non-epithelial tumors

In toto 22 non-epithelial tumors were encountered. They belonged to neoplasms of muscular & lymphatic tissues. None of nervous or vascular origin was seen.

a. **Leiomyomas** Three of the 8 belonging to this group (Table 2) were incidental findings during routine examination. Two were polypoid masses. Almost all were small. Four situated intramurally and originated from the muscularis propria. One (R-3709) was a large (10×10×10 cm), extragastric-growing pedunculated mass. In spite of the great dimension there was no sign of histologic malignancy. Because of the nature of the material and mode of examination, incidence of benign leiomyoma in this series appears to be too infrequent as compared with the figures reported by Meissner based on a thorough systemic

Table 2. Tumors of muscular origin.

	Age (years) & Sex	Site	Greatest dimension
Leiomyoma	41, 52, 68, 58, 55, 56, 32, 61, 68 m. m. m. m. m. m. f. m. m.	Fornix 3 Anthrum 7	0.01 cm—10 cm
Leiomyosarcoma	44, 63, 65, 67 f. m. f. f.	Fornix 4 Anthrum 0	3.0 cm—10.5 cm

examination of the autopsied stomachs (46% of 50 examined stomachs).

b. **Leiomyosarcomas** Findings on the four leiomyosarcomas are listed in theTable 3. The case 0-395 was reported in 1950 by Hoshino & Tanaka.[7] They were seen in 0.2% of all resected stomachs, 0.38% of all neoplastic lesions, and were encountered while 996 established carcinomas were resected. In the A. P. Stout's series[17] leiomyosarcoma comprises as high as 4.4 % of all gastric malignancies. This is due to lower incidence of common gastric cancers in the United States. The leiomyosarcomas represent about 30% of non-carcinomatous malignancies of the stomach, an incidence only second to the lymphoblastic series, and this is approximately comparable with the Stout's data[6] (33.4%). Occurrence in the higher age brackets and relative frequency in female (75%) are of note.

Remarkably they were often situated in the fornix and the anterior wall was predilected. Gross ulceration occurred twice. Preoperative diagnosis was possible in one case. One (case 0-2200) was combined with a separate medullary adenocarcinoma. All four showed intragastric growth. Although, in the case 0-1933 the tumor originated from the outer muscle layer, it remained intramural. None was extragastric.

As has been frequently discussed, histological differentiation of a leiomyosarcoma from its benign counterpart is often difficult. None had metastasis or involvement of the liver. In only one case regional nodes contained metastasis (case 0-395).

Table 3. Leiomysarcomas

Case	0-395	0-1933	0-2133	0-2200
Age & Sex	44 f.	63 m.	65 f.	67 m.
Size	7.0×9.0×2.3cm	8.0×10.5×6.5cm	1.0×10.0cm	3.0×3.5×5.0cm
Site	Ant. fornix	ant. fornix	ant. fornix	ant. fornix
Ulceration	+	++	++	−
Type of growth	intragsstric	intramural	intragastric	intragastric
Related musculature	musc. mucosae	musc. propria	musc. propria	musc. propria
Cellularity	+++	+++	+	++
Atypism	++	+++	+	+
Differentiation	+	++	+	+
Mitotic frequency	++	+++	++	±
Infiltrative tendency	+++	++	+	±
Metastasis	+ (node)	−	−	−
Vascular wall permeation	±	++	++	±

Criteria of malignancy applied are cellular atypism, mitotic frequency, and distinct tendency of invasion of the adjacent tissues, especially permeation of the vascular walls. Sometimes nuclear arrangement of regimentation type was noted, but there was no difficulty to exclude neurinomatous origin on the Mallory-stained sections. All of our cases are alive, one over 5 years.

Four autopsied cases of leiomyosarcoma of the stomach have been reported in the Japanese literature (Matsukuma 1939, Uehara[18] 1950, Iijima 1952, Fujita[4] & Okuyama 1952).

c. **Tumors of lymphoblastic origin** Of the nine tumors, invariably malignant, one was small-celled lymphosarcoma and the other eight were reticulum cell sarcomas. Three died within one year, and five were alive after one year. This group comprised 0.76% of all gastric malignancies and represented the most frequent sarcoma-type in the stomach, as among the series of Stout. No Hodgkin-type lesion, leukemic infiltration or plasmocytoma was encountered in this series.

Some of the small-celled medullary carcinomas of the stomach are extremely difficult to differentiate from the reticulum cell sarcoma. PAS and mucicarmin stains are often useful. The true alveolar structure of the carcinoma as compared with pseudoalveolar one of this group should be evaluated. It has been our experience that this needs considerable effort to review many sections taken from the lesions concerned. It is also possible that a portion of leiomyosarcoma may simulate lymphoblastic origin.

Table 4. Tumors of lymphoblastic origin.

	Age & Sex	Site	Dimension
0-26	46 f.	antrum	egg size
0-653	46 f.	fundus	6.5×6.0 cm
0-944	54 m.	fundus	13×6.2 cm
0-1091	53 m.	pylorus	9×11 cm
0-1482	56 m.	antrum	9.0×3.5 cm
0-1765	34 m.	antrum	8.0×5.0 cm
0-1863	64 m.	fundus	7.0×7.0 cm
0-2252	63 m.	fundus	6.0×5.5 cm
0-2414	61 m.	fundus	10.5×10 cm

d. **Miscellaneous conditions** Tumors of nervous origin, especially the benign and malignant neurilenmoma and paraganglioma, have been rather frequently reported in the world literature. There may be also gastric involvement in case of v. Recklinghausen's disease. None of them was seen in this series. Of note is that misinterpretation of leiomyomatous tumor as neurogenic has occurred in the earlier literature (cf. Golden-Stout, Minnes-Geschickter,[11] Smith[16] etc.).

Tumors of mesenchymal origin other than leiomyomatous and lymphatic groups are rare. Although there are some reports of lipomas, and angiomas, we have no experience of such tumors.

One case listed in the Table 1 (case 0-1329) contained a huge single mass of **malignant melanoma** occupying the cardia of a 57 year old male. It measured 10.8×8.0×6.5 cm. There was no lesion suggestive of its primary elsewhere. The patient died 9 months post total gastrectomy, apparently due to local recurrence. An autopsy was not done. In view of relative infrequeucy of malignant melanoma among the Japanese as compared with white population, this condition was thought to be extremely rare. Usually gastric metastasis of malignant melanoma is represented in multiple disseminated mucosal lesions.

By continuity extension of malignancies from such adjacent organs as the pancreas, liver, gall-bladder, adrenal and lymph-nodes, have been not infrequently experienced at the autopsy table. This surgical series contains none of this kind.

B. **Polypi**

The gastric polypi have recently been highly evaluated in conjunction with their premalignant nature. Twenty-eight cases with epithelial polypi were collected from this series of 1489 stomachs. Two polypous structures were not epithelial but leiomymatous. They were excluded from this subgroup, although certain grade of pseudoepitheliomatous hyperplasia was also present. An adenomyoma often protrudes into the lumen in shape of a polyp. But such has not been in the series. Polypous structures fully transformed into cancer, particularly in association with coexistent carcinoma of the other part of the stomach, were excluded, because of their possible metastatic origin.

As Spriggs has stressed, differentiation of polypi from the common hyperplastic gastritis is often too arbitrary. All dubious lesions were dropped from this series.

Two of the 28 cases were observed among 466 stomachs resected between 1946 & 1950. It composed only 0.43%, whereas from the more recent materials, between 1951 & 1955, as many as 26 polypous stomachs were seen among 1023 cases (2.54%). This is probably due to closer attention to the gastric polypi on the part of the surgeons in the recent years.

Of the 28 cases, 6 had multiple polypi: cases 0-1212, 0 1588, 0-1965 and 0-2344 had two polypi each ; case 0-2627 three, and case 0-2564 seven. No gastric polyposis was encountered. One of the multiple polypi was malignant in cases 0-1965, 0-2344 and 0-2564, respectively.

Table 6 shows age incidence of the gastric polypi. Frequent association of the polypi with higher age is shown.

As to the location, the polypi apparently predilect pyloric gland mucosa, but in 5 cases both the fundal and cardial gland areas were involved.

Table 5. Polyp

Case	Age & Sex	Site & dimension		Malignancy	Coexistant lesion	Metaplastic gastritis	
0-18	31 f.	pyloric canal	rice-grain	−	ulcer (heald)	fov.	+
0-351	65 m.	antrum	small fing.	−	adeno ca. medullare	intest.	++
0-1212	64 f.	fundus	2, ricegrain	−	adeno ca. scirrhosum	intest.	+++
0-1371	62 m.	pyloric canal	rice-grain	−	adeno ca. medullare	intest.	+++
0-1388	59 m.	antrum	1.5×1.5×1.5 cm	−		intest.	+
0-1572	65 m.	cardia	0.7×0.7×0.6 cm	−	adeno ca. medullare	intest.	+++
0-1588	58 f.	pylorus	2, 2×2 cm & rice-grain	−		intest.	+
0-1613	66 m.	antrum	rice-grain	−	adeno ca. medullare	intest.	+
0-1634	54 m.	pyloric canal	rice-grain	+		intest.	+
0-1878	56 m.	pyloric canal	rice-grain	−		intest.	+
0-1935	49 f.	fundus	0.8×0.8 cm	−		intest.	++
0-1965	72 f.	antrum	2, rice-grain	+	adeno ca. medullare	intest.	++
0-2003	63 m.	cardia	pea-sized	−	adeno ca. medullare	intest.	+
0-2024	46 m.	pylorus	rice-grain	−	adeno ca. papillotub.	intest.	+++
0-2086	49 f.	pylorus	0.6×0.6 cm	−	adeno ca. papillotub.	fov.	+
0-2091	62 m.	pyloric canal	0.8×0.8 cm	−	adeno ca. medullare	fov.	++
0-2161	54 m.	pyloric canal	finger-tip size	−	adeno ca. scirrhosum	intest.	+++
0-2294	49 m.	pyloric canal	0.7×0.7 cm	−	adeno ca. acinosum	intest.	+++
0-2324	56 f.	antrum	2, 0.8×0.8 & 1.2×1.2 cm	−		intest.	+++
0-2344	68 f.	pyloric canal	2, pea-sized & rice-grain	+	adeno ca. acinosum	intest.	+
0-2360	73 f.	antrum	1.0×0.7 cm	−	adeno ca. acinosum	intest.	++
0-2372	36 m.	antrum	1.5×1.5 cm	−		fov.	+++
0-2483	47 f.	antrum	1.0×0.7 cm	−		intest.	+
0-2514	64 f.	antrum	1.2×1.7 cm	−		intest.	+++
0-2527	42 m.	antrum	rice-grain	−	adeno ca. scirrhosum	intest.	+
0-2551	54 m.	pyloric canal	2×2.5 cm	+	adeno ca. scirrhosum	intest.	+
0-2564	62 m.	antrum & fundus	3 rice-grain sized & 4 pea-sized	+			
		pyloric canal	3, 0.6, 0.7, 0.8 cm			intest.	+
0-2627	49 f.		in diameter	−		intest.	+

Table 6. Age incidence of gastric polypi

Age	Number of cases with polyp	Total number of stomachs examined	Incidence
20–29	0	46	0
30–39	2	164	1.22%
40–49	7	400	1.75%
50–59	7	533	1.27%
60–69	10	279	3.58%
70–79	2	20	5.0%

Table 7. Concomitant metaplastic-gastritic changes of the mucosa in the stomach with polypi

		Number of cases	Malignant transformation
Intestinal metaplasia	+ (slight)	8	(4)
	++ (moderate)	3	(1)
	+++ (severe)	8	
Foveolar metaplasia	+	2	
	++	1	
	+++	1	

Histologically the following three histogenic types were recognized:

A. Hamartomatous polypi 5
   1. Pancreatic hamartia 3
   2. Pyloric gland adenoma 1
   3. Cardial gland adenoma 1

B. Hyperplaseogenic polypi 20
   1. Intestinal metaplastic type 15
   2. Foveolar epithelial type 5

C. Solid inflammatory polypi 3

A half of the polypi were pedunculated (14 cases) & the others were broad-based (14 cases). There was no correlation between histogenesis and gross appearance of the polypi: for example three hamartomas were pedunculated, whereas two broad-based.

Close association between the hyperplaseogenic and the metaplastic changes of the mucosal epithelium of the stomach is apparent. The condition has been called metaplastic gastritis by the authors. In 8 cases more or less severe intestinal metaplasia has occurred, whereas in 3 metaplasia of foveolar epithelial type was seen.

Five polypi (cases 0-1634, 0-2344, 0-2551 and 0-2564, and one of case 0-1965) showed

definitely autochthonous malignant transformation of the epithelium. Although four of the five cases had separate carcinomas in the distant part of the stomachs, the changes were believed to be early independent primary malignancies. The case 0-2564 had no established cancer elsewhere.

It is of interest, that all five polypi, which showed autochonous malignant transformation belonged to the hyperplaseogenic group. Two of them were associated with-hyperplastic metaplastic mucosa, whereas the other two with atrophic-metaplastic mucosa.

Concomitant lesions of the stomachs, for which the surgical resections were done, were carcinoma in 15 cases, and gastric ulcer in one case. Twelve cases showed neither carcinoma nor ulcer. Four polypi, in cancerous stomachs showed independent carcinomatous transformation of the epithelium.

Among others, Konjetzny stressed the meaning of polypi in the histogenesis of gastric cancer. Kuru[8] also showed diagramatically features of established cancers he believed to have originated in polypi. Detailed study of Murakami[12] on early histogenesis of cancer was also concerned with gastric polypi. Apparently gastric polypi are rather rare findings among average population. In the more recent series (Stewart 1931, Lawrence,[9] Rigler, et al, Buchstain, Spriggs et al, Yarnis[19] et al.) the incidence varies between 0.25 and 0.8% of several thousand autopsies. Borrmann[1] found in only 0.1% of a 11,475 consecutive autopsy series. They are said to be more frequent in association with pernicious anemia (Rigler-Kaplan),[15] but this is a remarkably uncommon condition in this country.

It should be commented from the above data that the gastric polypi are no rarity among our population, and that more attention should be paid to them, because at least the hyperplaseogenic subgroup, which comprises about 70% of all polypi, harbors potential danger of malignant transformation.

Summary

Data concerning the primary neoplastic lesions, excepting established carcinomas, found among 1489 surgical gastric series were presented. They consisted of 8 benign leiomyomas, one small-celled lymphosarcoma, 8 reticulum cell sarcomas, and 28 cases of epithelial polypi. One additional tumor was a huge solitary malignant melanoma without showing otherwise acceptable primary focus. The series contained 996 carcinomas and 343 ulcers. Histologic aspects of the non-carcinomatous tumors were discussed, and importance of gastric polypi as precancerous lesions of the stomach was emphasized.

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LEGENDS FOR THE PHOTOGRAPHS

Fig. 1. Leiomyosarcoma of the stomach at the anterior aspect, forming a fungating mass covered by the mucosa with exaggerated rugae. (0-395).

Fig. 2. Leiomyosarcoma (0-395). A typical spindle cell pattern with loose connective tissue stroma.

Fig. 3. Leiomyosarcoma (0-1933). Remarkable anaplasia simulating epithelial origin. Multiple mitotic figures are shown.

Fig. 4. Reticulum cell sarcoma of the stomach with a plateau-like elevation and a shallow ulcer in the center, fully simulating a carcinoma (0-1482).

Fig. 5. Reticulum cell sarcoma of the stomach (0-1765), involving the mucosa propria.

Fig. 6. Small celled lymphosarcoma of the stomach (0-944).

Fig. 7. Malignant melanoma of the stomach extending from the cardia to the lower part of the esophagus. No extragastric lesion suggesting its primary site was found (0-1329).

Fig. 8. Malignant melanoma (0-1329). Epitheloid arrangement in this particular region.

Some large cells contain melanin pigment.

Fig. 9. Two pedunculated benign polypi of the antrum (0-2324).

Fig. 10. Two larger benign polypi along the greater curvature situating side by side (0-2372).

Fig. 11. A broad-based polyp of the pyloric canal. The neighboring mucosa is rather atrophic (0-2024).

Fig. 12. Portion of a hyperplaseogenic polyp, showing intestinal epithelium (0-2614).

Fig. 13. A pedunculated polyp of the antrum (0-2483).

Fig. 14. Hyperplaseogenic polyp (0-1588), showing hyperplastic foveolar epithelium.

Fig. 15. A broad-based hamartomatous polyp of the stomach (0-2091). Pyloric gland type.

Fig. 16. Detail of Figure 6, showing pyloric gland adenoma.

Fig. 17. A large pedunculated polyp of the stomach (0-1388). It is composed of proliferating mucous gland ducts, which suggest pancreatic origin.

Fig. 18. Hamartomatous polyp showing pancreatic duct hamartia (0-1935).

Fig. 19. A papillary polyp (0-2551), measuring 2.0×2.0×2.5 cm, in the pyloric canal, a part of which showing autochthonous malignant transformation.

Fig. 20. Detail of Fig. 19, showing general benign adenomatous pattern of neck-epithelial type.

Fig. 21. Detail of Fig. 19, showing malignant papillary structures.

Fig. 22. An inflammatory polyp of the antrum. The polypous appearance is due to granulomatous changes in the deeper mucosal layers (0-2527).

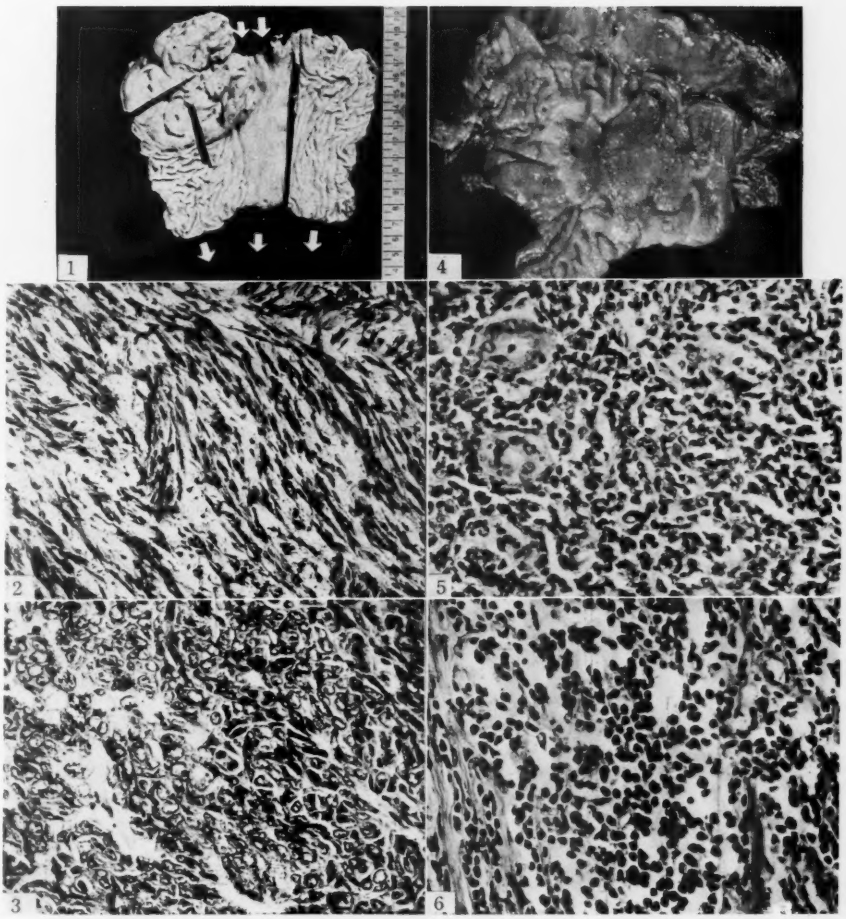
Fig. 23. Another inflammatory polyp (0-1572), showing granulation tissue and infiltration.

要　旨

胃の非癌腫性腫瘍：1489 例の切除胃についての組織学的及び統計的研究

松本昭三，太田邦夫 (癌研究所)

1947 年 9 月より 1955 年 11 月迄の 8 年間に，癌研究所病理部において検索された切除胃 1489 例の組織学的検索の結果から，胃癌を除いた胃腫瘍についての所見を綜括的に発表した。悪性腫瘍は，平滑筋肉腫 4 例，リンパ肉腫 1 例，細網肉腫 8 例で，良性腫瘍のポリープ 28 例，中には 5 例の悪性変性をみとめた。良性平滑筋腫は 8 例である。原発巣のなかった悪性黒色腫の 1 例は極めて稀れなものと考えられる。著者らは胃ポリープの内，過形成型は胃の前癌病変中，注目されるべき組織像を有すると考えている。外国文献について統計的に比較も試みた。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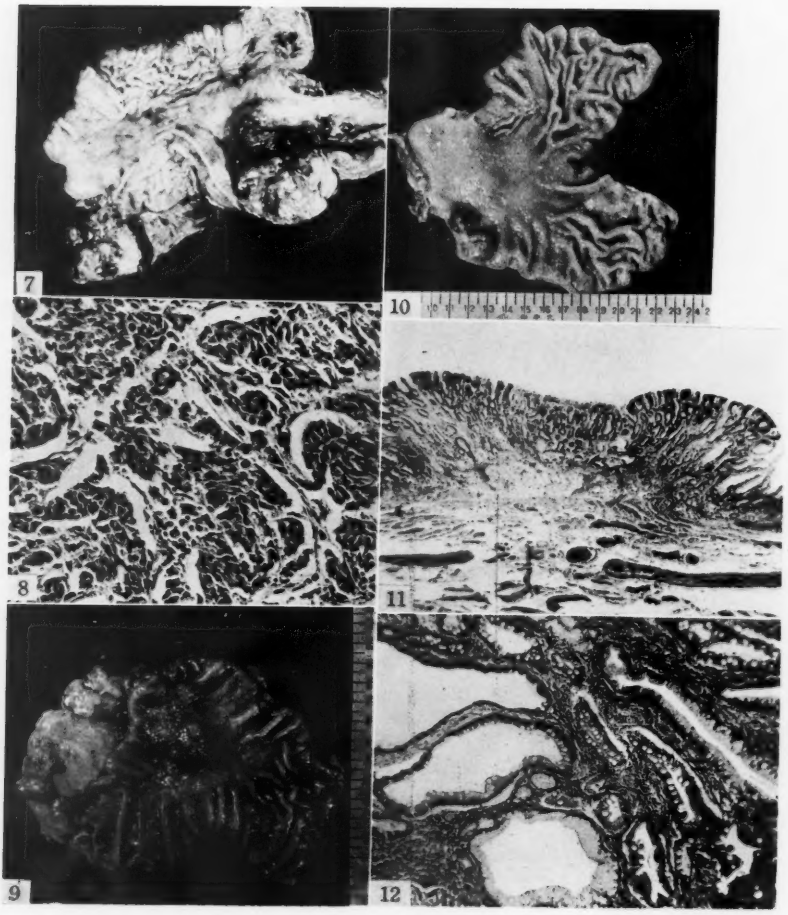
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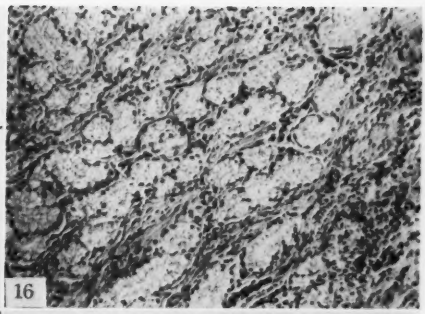
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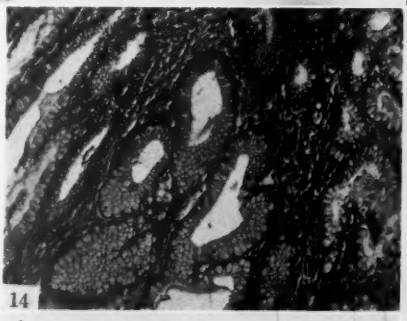
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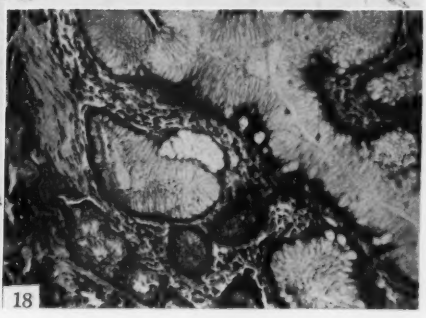
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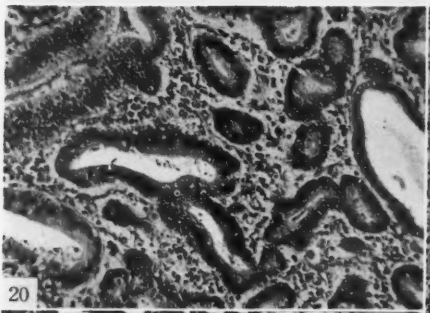
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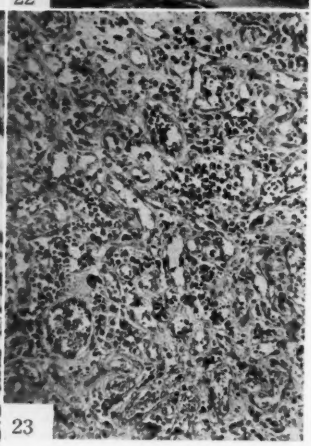
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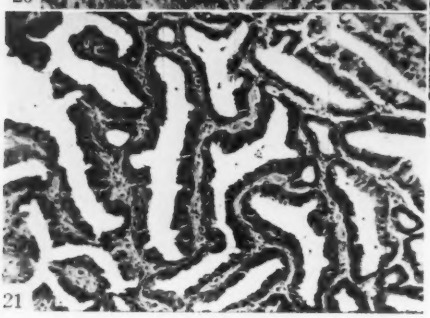
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[GANN, 47, 153~158; June, 1956]

SARCOMA PRODUCTION BY INJECTIONS OF ACRIDINE RED. A SUPPLEMENT TO EXPERIMENTAL STUDY OF XANTHENE DYES AS CARCINOGENIC AGENTS (With Plates XXXVII—XXXVIII)

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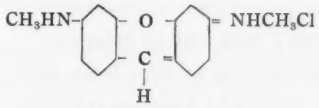
In a previous paper I (1) reported that certain xanthene dyes were carcinogenic for rats but not for mice. In these experiments, sarcoma was induced by repeated subcutaneous injections of the following dye stuffs: Rhodamine B, Rhodamine 6 G, Fluorescein Sodium, and Eosine Yellowish.

In the present experiment, the effect of Acridine Red injections was examined. Acridine Red is a kind of xanthene dye stuff, dimethyldiamino-xanthenyl chloride, (Colour Index No. 740; $C_{15}H_{15}N_2OCl$), forming brown powder, the watery solution of which has red color with a greenish-yellow fluorescene.



(Acridine Red)

Material and Methods

Experiment was started with 25 normal adult albino rats of a mixed Saitama strain, all weighing around 230 g. They were kept in wire cages in groups of four each, and were maintained on the usual laboratory diet of whole wheat with occasional supply of dried fish, cod-liver oil and green vegetables.

The preparation of Acridine Red used in the experiment was a product of the Tokyo Kasei Chemical Co., Ltd., Tokyo. The rats were given subcutaneous injections, at as nearly the same site as possible on the back, of 1 cc of 1 gdl waterly solution of Acridine Red once every week as a rule. After about 2 months of repeated injections, when the rats showed injury on the back, injections were reduced to once or twice monthly. After about 11 months, injections were increased to three or four times a month. Acridine Red solution was sterilized by heating before injection.

Results

Of the 25 rats, with which the experiment was started, 16 rats died early without showing tumor at the site of the injections. But one of them, dying on the 263rd day, had a cysticercus sarcoma (spindle cell sarcoma) of the liver. Out of the remaining 9, which lived 398 days or more, receiving 26 injections during the period, 7 rats produced sarcoma at the site of the injections. (Table 1)

Table I

Rat No.	Sex	Exp. days	Acridene Red		Body weight (end)	Tumor size (cm)	Histological diagnosis
			Total. mg.	Inject. No.			
1	f	416	270	27	270	4.2×3.9×2.7	Fibrosarcoma
2	m	416	290	29	270	No tumor	
3	m	417	270	27	272	1.8×1.2×0.5	Spindle cell sarcoma
4	m	469	330	33	275	3.8×2.7×2.6	Fibrosarcoma
5	m	476	370	37	220	No tumor	
6	m	487	300	30	214	1.8×1.5×1.0	Fibrosarcoma
7	f	503	370	37	151	3.9×3.4×2.6	Fibrosarcoma
8	f	506	440	44	180	1.5×1.0×0.8	Spindle cell sarcoma
9	f	563	470	47	185	2.5×2.5×0.9	Spindle cell sarcoma

Individual records of these 7 rats are as follows :

No. 1. A hard nodule of the size of thumb-tip was palpated on the 398th day, which attained the size of 2.1×1.6×1.5 cm by the 404th day. The tumor reached the size of 4.2×3.9×2.7 cm on the 416 th day. The animal was killed in very weakened condition on that day.

No. 3. A hard nodule of the size of the small-finger tip, first palpated on the 404th day, grew to the size of 1.8×1.2×0.5 cm on the 417th day. The animal died on that day.

No. 4. A thumb-tip sized nodule was palpated on the 411th day. The tumor reached the size of 2.7×2.0×1.6 cm on the 425th day, grew to the size of 3.1×2.2 ×1.7 cm on the 461 st day. The animal died on the 469th day with the tumor measuring 3.8×2.7×2.6 cm.

No. 6. On the 398th day a hard nodule of the size of the small-finger tip was found. The animal was killed in very weakened condition on the 487th day with the tumor measuring 1.8×1.5×1.0 cm.

No. 7. A hard nodule of the size of the small-finger tip, first palpated on the 452nd day, grew to the size of 2.7×2.3×1.8 cm on the 480 th day. The tumor reached the size of 3.9×3.4×2.6 cm on the 503rd day. The animal was killed in

very weakened condition on that day. Metastases were found in the lungs and left axillary lymph node.

No. 8. The tumor was found as a hard nodule of the size of small finger tip on the 446 th day. Remarkable ulceration surrounding the nodule was noted on the 461st day. The animal died on the 506th day with the tumor measuring 1.5×1.0×0.8 cm.

No. 9. On the 556th day a hard nodule of the size of small pea was palpated. The animal was killed on the 563rd day with the tumor surrounding ulceration measuring 2.5×2.5×0.9 cm. This rat also had a fibroadenoma of the mammary gland (2.8×1.9×1.1 cm) which may be regarded as spontaneous tumor.

In these 7 rats, subcutaneous connective tissue became thickened and small hard consolidations of various sizes became palpable during the 12 th to 14 th months. These consolidations gradually turned into irregular nodules (fibroma), which, then, rapidly grew into large tumors. The tumors were at first more or less diffuse, but as they increased in size they became clearly demarkated from the surrounding connective tissue. The tumor, once developed, grew very rapidly to a very large size, and soon killed the animal. However, in some cases the tumor showed superficial ulcer (Nos. 4, 6, 8 and 9) and invaded the adjacent muscle (No. 9).

Findings at Autopsy

All these tumors were found in the subcutaneous tissue at the site of the injections of Acridine Red, the shape being uneven semi-spherical to oblong with some irregularities. They were mostly elastic hard but some of them were softer. The cut surface was generally pearly white, occasionally with pinkish or redish areas due to haemorrhage, with hardly recognizable necrotic areas. The tumors were thinly encapsulated, clearly defined from the surrounding tissue to which they were slight adherent. The basal part of the tumor was tightly adherent to the underlying tissue. There was no evidence of the imbibition of the dye in the tumor tissue.

Macroscopic metastases were found in the lungs and left axillary lymph node (small finger tip size) (No. 7).

The liver was generally atrophic and histologically showed some hyperaemia and increase of Kupffer's stellate cells. Sometimes, vacuolar degeneration of liver cells was noted. No change in the nature of liver surface, color, or consistency was recognized in gross and no cirrhotic change was recognized histologically. Spleen showed increase of myeloid cell and splenic cell, hemosiderosis, fibrosis and hypertrophy of reticulum cells. Lymphnodes showed hypertrophy of reticulum cells and increase of lymphocytes. However, all these changes may be regarded

as of no special significance in connection with the local sarcoma production (except lung metastases of the tumor).

Transplantation

Attempts to transplant the tumor to normal rats were made in the cases of No. 1, No. 7 (metastasis in the left axillary lymph node) and No. 9.

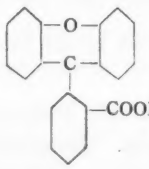
The transplantation from No. 1 took in 38% of the animals in the first generation, 14% in the second. Further transplantation was not made. The transplantation from No. 7 took in 80 % of the animals in the first generation, 100 % in the second, 100% in the third, 100% in the fourth, 100% in the fifth. The transplantation from No. 9 took in 100% of the animals in the first generation, 100% in the second. These transplanted tumors attained to a hens egg or even to an india-rubber ball size in two or three weeks, and the rats died of tumors in about one month on an average after transplantation. Microscopically, the transplanted tumors were classed as fibrosarcoma identical with the original tumors.

Histology of the Tumors

All tumors are diagnosed as fibrosarcoma, being composed chiefly of spindle cells. Mitosis were numerous in most tumors, and sometimes, round multinucliated giant cells were seen scattered through tumor tissues, and polymorphic cells too. Necrosis was found in varying extent in different tumors, and showed generally extensive central necrosis. The lymph node metastasis found in a rat coincided with the primary tumor in every histological feature.

Discussion

It has been shown that such water soluble xanthene dyes as rhodamine B, rhodamine 6 G, fluorescein sodium, and eosine yellowish produce sarcoma in rats by injections (1). These dyes all have o-carboxy-phenyl group and they produced sarcoma only in a small percent of the rats used.

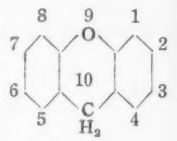


R=H, Na, C_2H_5 etc.

This time acridine red, lacking o-carboxy-phenyl, was tested with the result that is ultimately produced sarcomas at the site of injection in as many as 7 of the 9 rats that survived over 400 days, suggesting the possibility that the substances having no o-carboxy-phenyl show a higher carcinogenic activity than

substances having it. The period of production of tumor is about the same, however (2) (3) (4).

In this connection the results of injections of propylene glycol solution of xanthene may not be out of place.



(Xanthene)

This experiment was started with 12 rats; Xanthene was dissolved in propylene glycol at the concentration of 1.0 percent, and was injected subcutaneously on the back of the rats in 1.0 cc amounts. Injections were repeated one or two times a month, and were delivered into as nearly the same site as possible. After about 14.5 months, injections were increased to four or five times a month. 9 rats survived 300 days or more, receiving xanthene 110 mg, and propylene glycol 11 cc in 11 injections, one of the highest longevity surviving 621 days, receiving xanthene 420 mg, and propylene glycol 42 cc in 42 injections. None of the animals developed tumor at the site of the injection. Xanthene has no methylamino radical at the positions 2 and 7; and it seems probable that methylamino radical is necessary for carcinogenic activity of this class of substances.

However, as I stated previously, the exact mechanism of this type of sarcoma producing process is not clear at present, and there is as yet no indication even to tell whether physical or chemical reactions play the deciding role.

Summary

The carcinogenicity of a commercial sample of acridine red (a kind of xanthene dye stuff), administered by subcutaneous injection is reported. Sarcomas ultimately developed at the site of injection in 7 of 9 rats that received a total of 260 mg–470 mg of the dye in aqueous solution over a period of 398 days. One of them, killed on the 503rd day, had metastases in the lung and left axillary lymph node. No notable change in internal organs was found in the rats.

Acknowledgements

I take pleasure in acknowledging my indebtedness to Dr. Waro Nakahara, Dr. Makoto Tanaka and Dr. Shozo Matsumoto for their help and encouragement in the course of this work.

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Explanation of Figures

Plate XXXVII

Fig. 1. Rat No. 1, receiving 270 mg of acridine red in 27 injections, 416 days after the first injection.

Fig. 2. Rat No. 7, metastases in the lung, 503 days after the first injection.

Fig. 3. Metastasis found in axillary lymph node (rat No. 7).

Plate XXXVIII

Fig. 4. Histology of the lymph note metastasis in rat No. 7.

Fig. 5. Histology of the lung metastasis in rat No. 7.

Fig. 6. Original tumor in rat No. 1.

Fig. 7. A transplant from the original tumor of rat No. 1.

要　旨

Xanthene 色素による発癌実験（続報）

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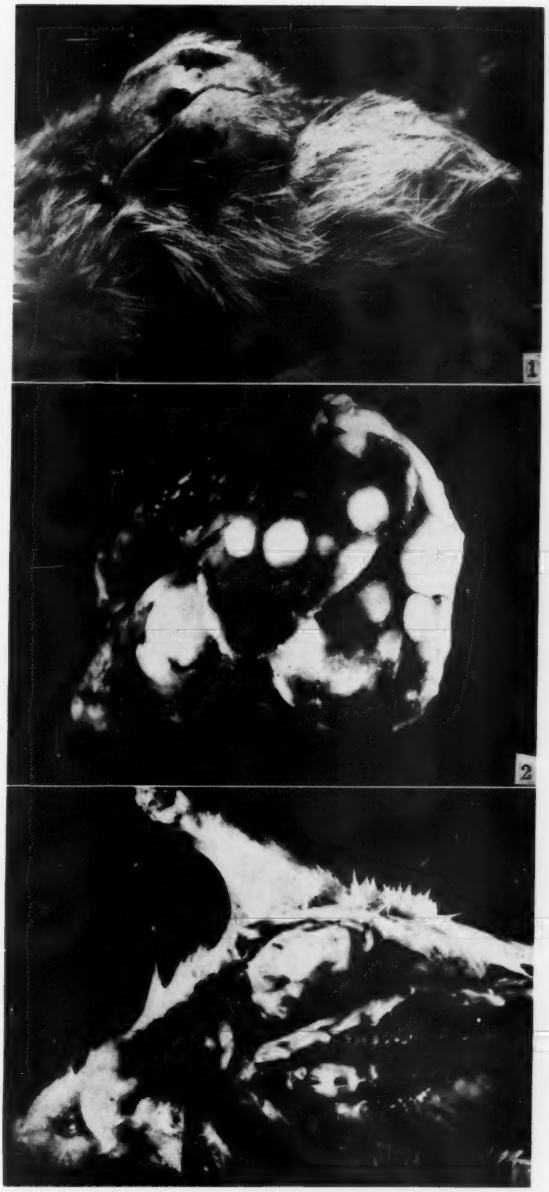
（癌研究所）

Xanthene 色素の一つである Acridine Red を用いて，白鼠の皮下に 1 gdl 水溶液を 1 週間 1 回宛，反復注射することにより，398 日以上生存した 9 匹の白鼠のうちの 7 匹に肉腫を発生せしめた。そのうちの 1 匹に肺臓及び淋巴腺の転移を認めた。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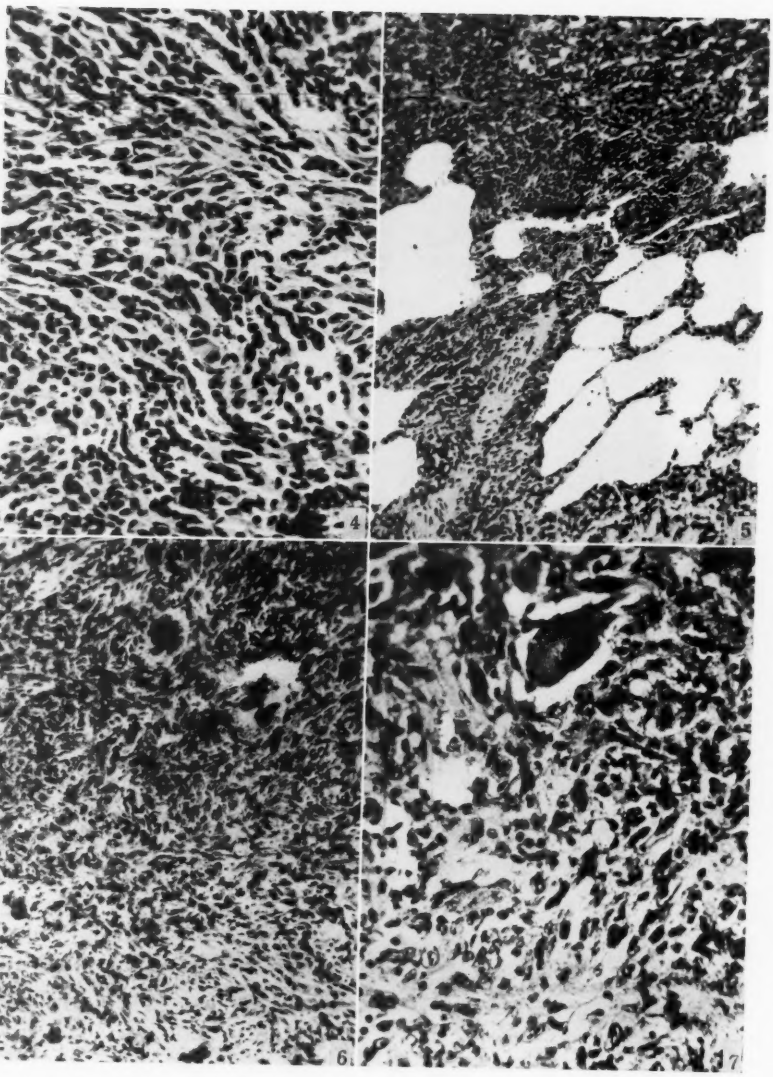
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[GANN, 47, 159~170; June, 1956]

THE MECHANISM OF LIVER CATALASE DEPRESSION BY 3-AMINO-1, 2, 4-TRIAZOLE

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It has been well established that the malignant tumor causes a marked depression of liver catalase of the host (1). Toxohormone, which Nakahara and Fukuoka succeeded in isolating from tumor tissues in 1948 causes a marked depression of liver catalase when it is injected into the normnl mice (2). Toxohormone injection brings about a marked decrease in liver catalase activity and not in blood catalase activity as in the case of tumor bearing animals. Concerning the mechanism of toxohormone effect on liver catalase, Fukuoka and Nakahara assumed it to be due to the disturbed synthesis of catalase (3).

Recently Heim *et al.* (4) observed that 3-amino-1, 2, 4-triazole (AT), which was known as the inhibitor of plant chlorophyll synthesis, caused remarkable depression in liver catalase and not in blood catalase when it was injected into rats. They pointed out that this effect of AT was similar to that of malignant tumor.

It was primarily in relation to the mode of action of toxohormone that the effect of AT on liver catalase was studied in this laboratory. In the course of these studies, it was found that AT action was completely different from toxohormone action, and further many interesting results were obtained about the mechanism of AT inhibition on liver catalase. These results are described in this paper.

Material and Methods

3 Amino-1, 2, 4-triazole (AT) was obtained from the laboratory of Tokyo Kasei Co., Ltd, Tokyo.

Crystalline catalase was prepared from cattle liver by the method of Shirakawa (5) and recrystallized three times. Crystalline catalase solution was diluted with 1/15 M phosphate buffer, pH 7.0. Throughout experiments, incubation was carried out at pH 7.0.

Catalase activity was measured gas-volumetrically by the method of Battelli and Stern (6) or by the method of Euler and Josephson (7). In the latter case, instead of permanganate titration, the colorimetric determination using titanium sulphate for hydrogen peroxide was applied (8).

Succinoxidase and cytochrome c oxidase were assayed by the method of Schneider and Potter (9). Liver porphyrin concentration was determined by the method of Schwartz *et al.* (10) with a slight modification.

As experimental animals, male rats (100-130 g) and male mice (16-30 g) were used. Liver homogenate was prepared using Potter-Elvehjem homogenizer with 30 to 40 volumes of 1/15 M phosphate buffer, pH 7.0. Supernatant which was obtained by centrifugation at 23,000 g for 30 minutes was used as "liver extract". AT was dissolved in 1/15 M phosphate buffer, pH 7.0, immediately before using.

Results

Data on liver catalase activity in AT injected mice are summarized in Table I. The liver catalase activity was found to have fallen to approximately 10 per cent of the normal when it was determined at three hours after injection (1 mg per g of body weight), in complete agreement with Heim's report.

Data on *in vitro* experiments of mouse liver homogenate are given in Table II. Final AT concentration was 1 mg per ml. Incubation at 0°C did not produce the inhibition of catalase activity at all, but that at 37°C inhibited almost completely the catalase activity for 3 hours.

Teble I. Liver Catalase Activity in Mice 3 hours after Injection of AT (1 mg/g)

Control	AT treated
11.6 ml	1.2 ml
10.8	1.3
12.4	0.4
15.3	1.1

Figures represent evolved oxygen by 1 ml of 2 per cent homogenate (Battelli and Stern's method).

Table II. *In Vitro* Experiments of Mouse Liver Homogenate with AT.

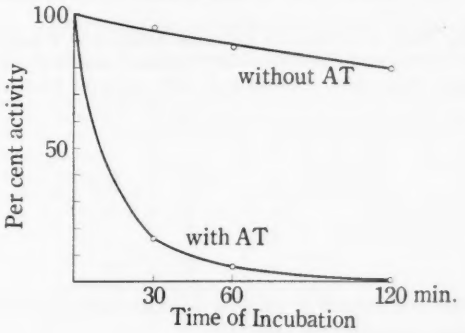
Time of incubation	Experiment A		Experiment B			
	0°C		0°C		37°C	
	without AT	with AT	without AT	with AT	without AT	with AT
0	40.0 ml	39.8	25.0	24.6	24.0	24.8
30 min	39.5	38.5	—	23.8	—	—
1 hr	40.6	39.2	—	—	—	—
2 hr	37.8	37.0	—	—	—	—
3 hr	—	—	24.4	—	16.4	0.1
						0.2

Reaction mixture: 30 times diluted homogenate 1 ml; AT 4mg/ml 1 ml, Figures represent evolved oxygen per 1.0 ml of reaction mixture.

It is obvious that AT inhibition takes place only in incubation at 37°C.

Time course of inhibition by AT is illustrated in Figure I. The activity decreased remarkably within 30 minutes when incubated with AT at 37°C. Control homogenate without AT showed a slower rate of decrease in activity during the two hours of incubation.

Fig. 1. Time Course of AT Inhibition.



The relation of AT concentration with inhibition rate is summarized in Table III. With final concentration of 0.5 mg per ml distinct inhibition took place. These results will be discussed later in this paper.

Next, the experiment using crystalline catalase was done. Contrary to the data obtained from the experiments using homogenate, crystalline catalase solution was not affected at all by AT either at 0°C or at 37°C. These results are given in Table IV. When the ***liver extract*** obtained by centrifugation at 23,000 g was added to the mixture of crystalline catalase solution and AT at 37°C, the AT inhibition occurred to the same extent as in the liver homogenate experiments.

Table III. Effect of Concentration of AT.

Final concentration of AT		Oxygen evolved	Per cent of activity
0	0	32.7	100
0.005 mg/ml	0.6×10^{-4}M	30.6	93.5
0.01	1.2 〃	27.8	85.2
0.05	0.6×10^{-3}M	25.8	78.7
0.1	1.2 〃	24.3	74.3
0.5	0.6×10^{-2}M	9.6	29.3
1.0	1.2×10^{-2}M	4.9	14.7
2.0	2.4×10^{-2}M	0.8	2.5

Reaction mixture: 30 times diluted homogenate of mouse liver, 4 ml; AT solution and water added to make the final volume 5 ml.

Incubation at 37°C for 1.5 hours.

Table IV. Crystalline Catalase and AT.

Temp.	Without AT	With AT
0°C	17.1 ml	18.4
37°C	23.5	21.8

Crystalline catalase solution diluted to suitable concentration, 3 ml; AT solution of 4 mg/ml or water, 1 ml. Incubation for 3 hours. 1 ml of reaction mixuture was assayed.

Table V. Combination of Crystalline Catalase Solution, AT and *Liver Extract.*

	Crystalline Catelase 2 ml	*Liver Extract* 1 ml	AT, 4 mg/ml 1 ml	Activity, Oxygen ml
A	+	−	−	29.7
B	−	+	−	2.5
C	+	−	+	29.1
D	+	+	+	1.0
E	+	+	−	19.4
F	−	+	+	0.0

Crystalline catalase was diluted to suitable concentration. Incubation at 37°C for 2 hours. 1 ml of reaction mixture was assayed.

The data of experiments on the combination of crystalline catalase solution and *liver extract* are given in Table V.

In Table V, the crystalline catalase solution with and without AT showed the same activity (A & C). If *liver extract* with low catalase activity (B) was added to mixture of crystalline catalase and AT, the activity was lost completely (D). The fact is known that when *liver extract* is added to crystalline catalase, (E) the latter's activity is decreased as already reported by Endo, Sugimura, Ono and Konno (11) and many other workers (12, 13, 14).

The possibility was examined that *liver extract* metabolized AT at 37°C and changed it into an inhibitive substance in regard to catalase. In Tables VI and VII, the data on these experiments are given. As shown in Table VI, where liver homogenate of control mouse and AT injected mouse were combined and incubated at 0°C for 30 minutes, inhibition was not observed at all. The attempt

Table VI. Absence of AT Metabolite having Inhbitive Action of Catalase.

Control mouse	AT treated mouse	Combination
No. 1 40.0 ml	No. 3 2.4	No. 1+No. 3 41.8
No. 2 23.8	No. 4 1.9	No. 2+No. 4 23.0

0.5 ml of 30 times diluted homogenate was assayd.
Mice were killed 3 hours after injection ot AT (1 mg/g).
Figures represent evolved oxygen.
Combined homogenate was incubated for 30 minutes at 0°C.

to ascertain the occurrence of the inhibitive metabolite *in vitro* also failed. Data on *in vitro* experiments are given in Table VII.

AT action on catalase of various cellular fractions was next studied, with the results given in Table VIII. Cellular fractions were obtained by the procedure of Shneider and Hogeboom. Nuclear, mitochondrial, microsomal and supernatant fractions were treated with AT. The degree of inhibition was greater in microsome and supernatant than in mitochondria and nuclei. It was presumed, therefore, that the *liver extract* factor which is necessary for AT inhibition of the catalase is contained mainly in microsome and supernatant fractions.

Table VII. Absence of AT Metabolite having Inhibitive Action on Catalase.

20× rat liver homogenate	10× rat liver homogenate treated with AT (1)	Water	Final AT equivalent concentration	Oxygen ml (2)	k (3)
1.5 ml	0 ml	1.5 ml	0 mg/ml	31.0	0.1035
1.5	0.3	1.2	1.0	33.5	0.1080
1.5	1.0	0.5	3.3	—	0.1025
1.5	1.5	0	5.0	33.0	0.1010

(1) 10 times diluted rat liver homogenate incubated with AT of 10 mg/ml concentration for 2 hours at 37°C.

Non-treated liver homogenate and AT treated liver homogenate were mixed and incubated for 2 hours at 0°C.

(2) Evolved oxygen per 1.5 ml of reaction mixture by the method of Battelli and Stern.

(3) k obtained by the method of Euler and Josephson using aliquots ot reaction mixture.

Table VIII. Effect of AT on Cellular Fractions.

	Control	AT treated	Per cent activity
Nuclei	0.0472	0.0113	23.9
Mitochondria	0.6170	0.2640	67.0
Microsome	0.2800	0.0099	3.5
Supernatant	0.4720	0.0333	7.2

1 g rat liver was fractionated, and nuclei, mitochondria, microsomes, were resuspended in 10 ml of buffer. They were treated with AT of final concentration of 1 mg per ml for 2 hours at 37°C. Supernatant was diluted to 15 ml and treated with above described conditions. Figures represent k obtained from the method of Euler and Josephoson per original liver of wet weight 100 mg equivalent.

Next, the dialyzed and boiled *liver extract* were prepared and tested in order to find out the characteristics of *liver extract*. Dialyzation was done against 1/15 M phophate buffer, pH 7.0, for 24 hours. Data of these experiments are tabulated in Table IX, from which it is clear that boiled or dialyzed *liver extract*

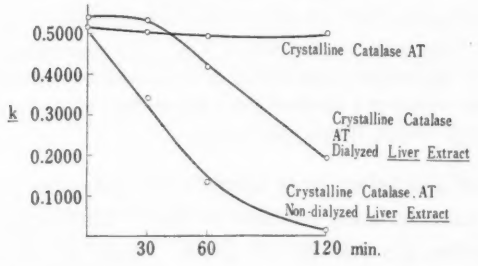
cannot develop AT action. When the two *extracts* were combined, inhibitive action of AT was restored. The original *liver extract* or boiled *extract* showed by themselves a slight inhibitive action on crystalline catalase solution (B, D). This fact agrees with the findings already reported concerning catalase depressing tissue factors (11). That dialyzed *liver extract* displayed no inhibition on crystalline catalase solution (F), well agreed with the findings by Hargreave and Deutsch (12) and Putzer *et al.* (16), and this means that the catalase depressing tissue factor may be dialyzable. In conclusion, it is evident that the *liver extract* is composed of two components, namely, heat labile, non dialyzable protein (s) (enzyme (s)) and heat stable, dialyzable cofactor (s).

Table IX. Experiments of Whole *Liver Extract*, Boiled *Liver Extract* and Dialyzed *Liver Extract*.

	Crystalline Catalase Solution 2 ml	Whole *Liver Extract* 1 ml	Dialyzed *Liver Extract* 1 ml	Bioled *Liver Extract* 1 ml	AT 5 mg/ml	Catalass Activity 0_2 ml
A	+	−	−	−	−	17.2
B	+	+	−	−	−	13.6
C	+	+	−	−	+	2.0
D	+	−	−	+	−	13.4
E	+	−	−	+	+	13.9
F	+	−	+	−	−	21.4
G	+	−	+	−	+	14.9
H	+	−	+	+	+	0.0
I	+	−	+(0.5 ml)	+(0.5 ml)	+	0.0
J	+	−	−	−	+	21.0

Final volume, 5 ml: Final AT concentration 1 mg/ml
Incubation for 1 hour at 37°C.
1 ml of reaction mixture was assayed.

Figure 2. Time Course of AT Inhibition with Dialyzed and Non-dialyzed *Liver Extract*.



Expermental conditions were the same as described under Table IX.

The action of boiled ***liver extract*** on the catalase inhibition with AT and dialyzed ***liver extract*** was not replaced by Mg^{++} and Ca^{++}, nor by any of the many intermediates, such as fructose diphosphate, lactate, glucose, pyruvate, citrate, succinate, malate and glutamate.

The time courses of AT inhibition on the crystalline catalase solution with non-dialyzed and dialyzed ***liver extracts*** are illustrated in Figure 2. In long time incubation, dialyzed enzyme also produces an inhibition though to a lesser degree than in the case of the non-dialyzed ***liver extract.***

Next, we attempted to test in one hour incubation experiment, whether nucleotide cofactor can replace the boiled ***liver extract.*** At first, coenzyme A, diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) were tested, and strangely enough any of the three cofactors was found to be capable of replacing the boiled ***extract.*** This finding was confirmed by repeated experiments. Since the component common to coenzyme A, DPN and ATP is adenine, the various adenine related compounds were tested. Typical data are given in Table X, which show that CoA, ATP, DPN, AMP, adenosine, adenine, hypoxanthine, DNA are all active. Uracil has not the action of replacing of boiled ***extract.*** Although any interpretation has not been given for these results at present, they offer a

Table. X. The Effect of Various Cofactors.

Addition		*k*
None	Crystalline Catalase Solution	0.3660 (0.3780)
None	Crystalline Catalase Solution, Non dialyzed *Liver extract*	0.0025 (0.2775)
None	Crystalline Catalase Solution Dialyzed *Liver extract*	0.2780 (0.5120)
ATP, 4 mg	〃	0.0275 (0.4800)
AMP, 4 mg	〃	0.0000 (0.4420)
Adenosine, 4 mg	〃	0.0000 (0.4420)
Adenine, 4 mg	〃	0.1900 (0.5200)
CoA, 4 mg	〃	0.0065 (0.4865)
DPN, 1 mg	〃	0.0195 (0.4775)
Hypoxanthine, 4 mg	〃	0.0030 (0.4345)
DNA, 10 mg	〃	0.0150 (0.4330)
Uracil, 4 mg	〃	0.3090 (0.4885)

Reaction mixture: 1 ml of crystalline catalase solution of suitable concentration; 1 ml of AT (5 mg per ml); *Liver extract* or dialyzed *liver extract*, 1 ml; Addition 1 ml. Final volume, 5 ml; *k* is obtained from Euler and Josephson's method with aliquot of reation mixture, incubation for 1 hour at 37°C Figures in the bracket represent *k* of reaction mixture without AT. DPN, 95 per cent purity, was obtained form Sigma Chemical Company, U.S.A. Coenzyme A, 8 units per mg, was prepared from hog liver by the method of Lipmann et al. (17) (in the first step of acetone precipitation)

very interesting problem for future investigation.

It is noteworthy that the AT inhibition on liver homogenate catalase does not appear under the anaerobic condition using Thunberg's tube. As shown in Table XI, the difference between the results of anaerobic and aerobic incubations was very distinct.

It is of interest to investigate whether AT affects other iron porphyrin enzymes and porphyrin metabolism in liver. Data about hepatic cytochrome c oxidase are given in Table XII. AT depressed almost completely catalase activity in 3 hours after injection of it into mice, but no significant effect upon cytochrome c oxidase could be observed. Further, succinoxidase activity was not affected by AT injection. When incubated ***in vitro*** with AT at 37°C for 2 hours, homogenate of mouse liver did not show the depression of liver cytochrome c oxidase. Although the colorimetric method for cytochrome c oxidase reported by Straus (18) had been used in some parts of these experiments, the results obtained did not differ.

As shown in Table XIII hepatic porphyrin concentration after adminstration of AT was not so remarkable. Recently, Schwartz *et al.* (19) (20) described the remarkable depression of hepatic catalase, the increase of hepatic porphyrin concentration and the occurrence of hepatic "green porphyrin" in the Sedormid treated rats. In our experiments as described above AT produced the depression

Table XI. Experiments under Aerobic and Anaerobic Conditions.

Experiment A

Time of Incubation	AT	Condition	k (per 25 mg wet weight)
1 hr	—	aerobic	0.7300
1 hr	+	aerobic	0.0340
2 hrs	—	aerobic	0.7230
2 hrs	+	aerobic	0.0160
1 hr	—	anaerobic	0.6580
1 hr	+	anaerobic	0.5820
2 hrs	—	anaerobic	0.7420
2 hrs	+	anaerobic	0.6810

30 times diluted homogenate of rat liver, 3 ml: 4 mg/ml AT, 1 ml. Incubated at 37°C.

Experiment B

Crystalline Catalase Solution 2 ml	*Liver Extract* 1 ml	AT 4 mg/ml1, ml	Condition	Catalase Activity, ml
+	—	—	aerobic	28.0
+	+	—	aerobic	23.1
+	+	+	aerobic	0.0
+	+	+	anaerobic	11.3

Incubation for 2 hours at 37°C. 0.5 ml of reaction mixture was assayed.

Table XII. Effect of AT on Cytochrome c Oxidase and Succinoxidase (*in vivo*).

	Cytochrome c Oxiease*	Succinoxidase*	Catalase Activity	
			k (per 10 mg wet weight)	0_2 ml (per 10 mg wet weight)
Control	42.0	—	0.2310	—
Control	42.0	—	0.1630	—
Control	42.8	—	0.1875	—
Control	—	22.4	—	26.5
AT treated	42.8	—	0.0660	—
AT treated	29.2	—	0.0070	—
AT treated	34.0	—	0.0040	—
AT treated	—	31.2	—	2.4

* Data are expressed as μl O_2 consumption per hour per mg of wet tissue weight in cytochrome c oxidase and succinoxidase.

AT was injected into mice at the rate of 1 mg/g. Animals were killed 3 hours after injection.

Table XIII. Effect of AT Injection on Porphyrin Concentration of Liver.

Injected Dose of AT	Time from last injection to sacrificing	Porphyrin concentration ug per g	Catalase activity k (per 10 mg wet weight)	Green Porphyrin fraction	
				E_{380}	E_{400}
Two injections of 0.5 mg/g each with	12 hrs	0.265	—	—	—
12 hours interval	12 hrs	0.215	—	—	—
None	—	0.188	—	—	—
None	—	0.176	—	—	—
Four injections of 1 mg/g each with	24 hrs	0.181	0.0480	0.185	0.174
24 hours interval	24 hrs	0.250	0.0360	0.322	0.242
Three injections of 1 mg/g each with 24 hours interval	18 hrs	0.200	0.0210	0.250	0.200
None	—	0.170	0.3480	0.186	0.143
None	—	0.169	0.3170	0.216	0.165
None	—	0.167	0.2000	0.207	0.168

Green porphyrin fraction in 6.5 ml of 7 N HCl was obtained by the procedure of Schwartz and Ikeda (20). Figures represent the extinction per 10 g liver with Beckman spectrophotometer at 380 mμ and 400 mμ.

of liver catalase but not the increase of hepatic porphyrin nor the occurrence of hepatic "green porphyrin."

Discussion

The data presented above show that ***liver extract*** containing enzyme (s) and cofactor (s) are necessary for AT inhibition of hepatic catalase, while it is also

necessary that the temperature is kept at 37°C and that oxygen is present.

AT action is similar to toxohormone action on the point that they produce the depression of hepatic catalase and not the depression of blood catalase after the injection into animals. But inasmuch as toxohormone does not have the action *in vitro* upon existing catalase (2) (11), it is obvious that it has the entirely different mechanism from AT.

Schwartz *et al.* (19) (20) reported that administration of sedromid (allylisopropylacetylcarbamide) to rats produced a marked and rapid fall in liver catalase activity, and simultaneously, a great increase in hepatic prophyrin concentration. They observed no significant changes in erythrocyte catalase activity nor in the activity of liver cytochrome c oxidase and succinic dehydrogenase. AT action is similar to sedormid action, but the administration of AT did not produce great increase of hepatic porphyrin concentration. Because "green porphyrin" was not observed in AT treated animals, the occurrence of "green porphyrin" in sedormid treated animals may not be the result of the depression of catalase activity, but may be related to the inhibition of catalase synthesis. Injections of toxohormone produce appreciable increase in hepatic porphyrin hand in hand with the well known depression of liver catalase (21).

Regarding the mode of action of AT, two possibilities are considered. One possibility is that AT is changed metabolically by the enzyme(s) and co-enzyme(s) contained in *liver extract* into the inhibitive substance, which immediately combines with the available catalase molecules. This inhibitive substance is considered to be very labile, as it is not recognized by the usual method in incubated mixture. The fact that oxygen is required would suggest that metabolic change of AT involves an oxidation, and this facts would also support the possibility mentioned above. Since AT developed the rather unstable color, having a maximum absorption at 500 mμ with Bratton-Marshall's reagent, an attempt was made to determine the metabolic change of AT. It was found only that when homogenate with AT at concentration of 1 mg per ml was incubated for 2 hours at 37°C, AT concentration decreased to 0.84 mg per ml.

Another possibility is that AT modifies the protein of catalase molecule and slightly denatures it, which has in itself the full activity but is easily attacked by proteinase such as cathepsin contained in *liver extract.* It is already reported that the proteinase more easily digests the denatured protein than the intact protein molecules (22). With this assumption, we can hardly explain the requirement of oxygen although Yanagita *et al.* reported that trypsin more easily digests the urea-denaturated haemoglobin under aerobic condition than anaerobic condition (23). Furthermore, it must be pointed out that higher concentration of substance is generally required for protein denaturation than in the case of AT.

Up to now, no explanation for the requirement of adenine compounds has been obtained, but it is probable that they accelerate the interactions among AT molecules, catalase and ***liver extract*** enzyme(s).

Summary

1. 3-amino-1, 2, 4-triazole (AT) depressed remarkably the liver catalase after the injection into mice and rats as reported by Heim *et al.*

2. AT action on catalase activity was also recognized in ***in vitro*** experiments. AT, ***liver extract*** and catalase must be incubated together at 37°C for the demonstration of the inhibiting action.

3. Oxygen was also found necessary for the AT action.

4. ***Liver extract*** contained two components, namely, heat labile, non-dialyzable proteins (enzymes) and heat stable, dialyzable cofactor(s). Cofactor(s) can be replaced by adenine containing compounds and hypoxanthine.

5. AT caused no significant effect on cytochrome c oxidase and no great disturbance of porphyrin metabolism of liver, the latter fact probably indicating that the AT effect is not based on the disturbance of catalase synthesis.

6. The action of AT was found to be completely different from that of toxohormone.

7. Two possibilities on the mechanism of AT action were considered and discussed.

The author wishes to acknowledge the encouragement of Dr. W. Nakahara, Director of Cancer Institute, during the course of this work and to thank Dr. Y. Ogura, Botanical Institute, Tokyo University, and Dr. T. Ono, Cancer Institute, for their kind suggestions.

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要　旨

3-amino-1, 2, 4-triazole にする肝カタラーゼ低下の機構

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3-amino,-1 2, 4 triazole (AT) は動物体重 1g 当り 1mg の注射で，肝カタラーゼを極度に低下させることが出来る。この際赤血球カタラーゼには影響がない。この現象は担癌動物およびトキソホルモン注射動物に見られる所と同一であるので，トキソホルモン作用の機構追求と関連して本問題の研究を行った。その結果 AT はトキソホルモンと全く異る作用機作を持つとともにそのカタラーゼ阻害機構に関して非常に興味ある所見を得た。

AT は ***in vitro*** で 37°C で肝ホモジエネートと incubate すると全く肝カタラーゼを阻害する。結晶カタラーゼ溶液は 37°C で AT と incubate しても阻害されないが，これに肝抽出液を少量加えると完全阻害が起る。この肝抽出液は heat labile, 非透折性の酵素蛋白と，耐熱性透折性の助因子とからなり後者はアデニン化合物で代用される。この阻害作用発現には酸素の存在が必要である。AT が肝酵素によりカタラーゼ阻害物質に変化すると考えられるがそのものを証明することは出来ないので，不安定なものと考えられる。

AT は ***in vivo*** および ***in vitro*** でチトクローム C 酸化酵素に作用しない。また動物注射で肝カタラーゼを極度に低下させた場合でも肝のポルフイリン代謝に大きな影響はおよぼさない。

[GANN, 47, 171~180; June, 1956]

PORPHYRIN METABOLISM IN TUMOR BEARING ANIMALS. FREE PORPHYRIN IN LIVER, HARDERIAN GLAND AND URINE AND THE EFFECT THEREON OF TOXOHORMONE

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The marked decrease of catalase activity in the liver of tumor bearing animals has been accepted as the most universal and tumor specific phenomenon in tumor-host problems (1). Nakahara and Fukuoka (2, 3) have contributed in disclosing the mechanism of this phenomenon by revealing the existence in tumor tissue of a catalase depressing factor, named by them as "toxohormone", by actually extracting and concentrating the substance in the form of potent fractions. As to its mode of action, they offered a hypothesis deduced from their own experimental results that it may injure the synthesis of that enzyme in liver by impairing the iron utilization. From this point of view, it was suggested that toxohormone may exert a similar effect on the synthesis of other hemoproteins, i. e., hemoglobin and cytochrome systems, and may thus be responsible for the major part of the systemic effect in neoplastic disease (4).

In the case of lead poisoning, it was postulated by Vanotti (5) that lead affects the mechanism of the iron incorporation into protoporphyrin in the sequence of hemoprotein synthesis and causes erythrocytic porphyria (6, 7). If we accept Nakahara and Fukuoka's above hypothesis, it is reasonable to expect a porphyric state in tumor bearing animals, but since there is no data concerning the porphyrin metabolism of tumor bearing animal, it is important to accumulate the necessary information bearing upon the subject.

As reported in the preliminary paper, which appeared in this journal, it was already ascertained in this laboratory that the free porphyrin of the erythrocyte is increased in the tumor bearing animals, which have anemia(8). In the present paper, the porphyrin levels of liver, Harderian glands and urine of tumor bearing rats, and the effects of toxohormone on them are reported.

EXPERIMENTAL

The general experimental conditions were the same as those of the first report (8), that is, the animals used were male white rats of Saitama mixed strain, the tumor implanted was rhodamine sarcoma (Umeda-fibrosarcoma) and the toxohormone employed was obtained from the same tumor by the procedure of Nakahara

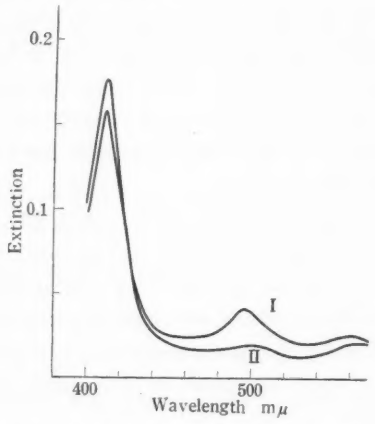
and Fukuoka (2), which was used in 300–400 mg doses confirmed to be enough to cause a marked liver catalase depression for rat of about 150 g body weight.

Liver Porphyrin

In connection with the remarkable depression of catalase activity in liver by tumor implantation and/or toxohormone injection, the porphyrin level in this organ was examined in the first place.

The quantitative analysis of porphyrin in the tissue was carried out according to the method of Schwartz et al. (9, 10), and in the case of liver about 5 g of wet tissue was subjected to this procedure. Among the uro-, copro- and protoporphyrin fractions from the each liver of normal, rhodamine sarcoma bearing as well as toxohormone injected rats, only the last fraction showed definite red fluorescence by near ultraviolet light (Hanau-ultraviolet lamp) excitation.

Fig. 1
The absorption curves of liver protoporphyrin fraction.



I. Rhodamine sarcoma bearing rat
II. Normal rat

The absorption curve of protoporphyrin fraction is illustrated in Fig. 1. As one can see in the figure, the curve of normal rat liver is closely in accordance with that of pure protoporphyrin in concentrated HCl solution, having the absorption maximum at 411.5 mμ, which is characteristic to the Soret band of this porphyrin. Although, those from the liver of tumor bearing and toxohormone injected ones show the same characteristic Soret band as in normal animal, they contain beside it an unexpected absorption peak at 493 mμ which is probably due not to porphyrin. The nature of the material responsible for 493 mμ absorption will be discussed in the later section. But practically it can be ascertained that the two peaks, that is at 411.5 mμ and at 493 mμ, are separated from each other. Therefore the protoporphyrin concentration in the extract was determined at 411.5 mμ by Beckman spectrophotometer, and at the same time, the extinction at 493 mμ was checked for comparison.

Schmid and Schwartz reported the occurrence of "green porphyrin" in the liver of Sedormid treated animal, and the most attractive description in their reports is that there exists a close relation between liver catalase depression and the increase of "green porphyrin" by the treatment. The so called "green porphyrin" levels in the liver of the tumor bearing, toxohormone injected as well

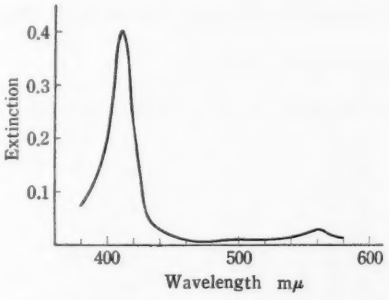
as normal rats were therefore pursued by the procedure of Schmid and Schwartz (11), which consists of 7.5 N HCl extraction from the ethylacetate : acetic acid extract of tissue after removing the other porphyrins by the ordinary procedure. This green porphyrin fraction was washed with ethylacetate twice to remove the trace of hemin contamination, and was examined as to its absorption curve.

Porphyrin in Harderian Gland

The concentration of protoporphyrin in the Harderian gland is extraordinarily high, and as revealed by the quantitative determination, its content accounts for an appreciable portion of the total porphyrin in rat body. And although its physiological function is not yet clarified, Figge has pointed out that external porphyrin is accumulated in this gland, and its porphyrin level reflects the states of porphyrin metabolism in the other organs (12).

So the examination of porphyrin content of this gland was considered to be useful for the purpose of detecting the disturbance of porphyrin metabolism in tumor bearing as well as toxohormone injected animals. By the preliminary experiment, the kind of porphyrin in this gland was revealed to be composed exclusively of protoporphyrin, and this was comfirmed by the observation of the absorption curve, which is illustrated in Fig. 2. The same procedure as for liver was adopted in the quantitative analysis of this gland.

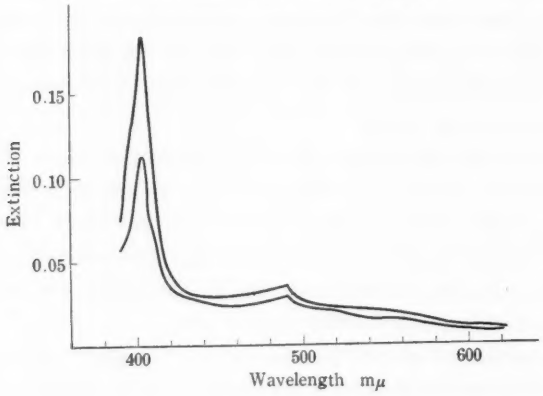
Fig. 2. The absorption curve of protoporphyrin fraction from Harderian gland of normal rat.



Porphyrin Excreted in Urine

The determination of porphyrin in urine was carried out according to the procedure of Schwartz et al. (13). Coproporphyrin was the sole kind of porphyrin which was detected in the urine of normal as well as rhodamine sarcoma bearing and/or toxohormone injected rats. The absorption curves of coproporphyrin fractions extracted from the normal and tumor bearing rats are illustrated in Fig. 3, which show the typical Soret band as coproporphyrin. But those obtained with the urine of tumor bearing rats contained the extra absorption maximum at 490 mμ, which is supposed to be due to the same substance as noted in liver protoporphyrin fraction. Since there was no crystalline coproporphyrin sample available as standard, the concentration in each sample of urine was expressed by the extinction at the absorption maximum (405 mμ).

Fig. 3. The absorption curves of coproporphyrin fraction (urine) from rhodamine sarcoma bearing rat.



Results

Porphyrin Level in Liver

The protoporphyrin content in the liver and the optical density of the protoporphyrin fractions at 493 mμ are tabulated in Table 1, and the individual data of the former are plotted in Fig. 4 with the columns to indicate the average values for each group. As one can see from these data, in the liver of tumor bearing rats, the increase of protoporphyrin concentration and the extinction at 493 mμ was very remarkable and the increment of the former in average was 87.5 per cent

Table 1 Protoporphyrin concentration in liver (μg/100 g)

No. of rat	Normal	Rhodamine sarcoma	Toxohormone injected
1	20.9	33.8	21.2
2	18.4	20.0	18.5
3	17.8	23.2	17.6
4	8.4	25.4	26.2
5	21.1	23.3	29.7
6	24.2	42.2	22.6
7	17.1	33.1	22.0
8	20.1	34.2	23.3
9	12.1	34.2	17.1
10	16.5		
11	16.5		
12	7.6		
13	8.0		
Mean	16.0	30.0	22.1

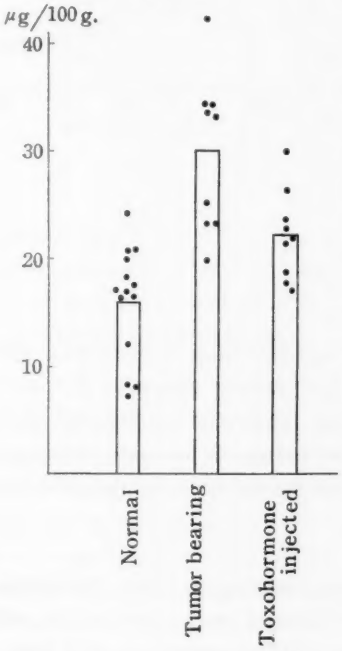
Extinction of protoporphyrin fraction at 493 mμ

No. of rat	Normal	Rhodamine sarcoma
2	0.020	0.042
3	—	0.073
4	0.016	0.097
5	0.040	0.028
6	0.023	0.070
7	0.017	0.063
8	0.012	
9	0.017	
10	0.030	
Mean	0.022	0.062

of the normal value (from 16 μg/100 g wet weight to 30 μg/100 g). Toxohormone injection also causes a significant increase in protoporphyrin level of liver, and the average in this group was 22.1 μg/100 g, which amounted to about 40 per cent over that of normal.

The absorption curves of the green porphyrin fraction are illustrated in Fig. 5: a) normal rat liver, b) rhodamine sarcoma bearing, c) toxohormone injected. To a great surprise, that from normal rat liver contains only a negligible peak at 493 mμ, those from tumor bearing or toxohormone injected ones exhibit a definite absorption peak at this wavelength. In one of the extreme cases in the toxohormone injected group, this peak exceeded that of the shorter wavelength. The absorption maximum at the shorter wavelength located around 380 mμ, is slightly different from that of the green porphyrins described by Schwartz (11), which has the maxima ranging from 385 mμ–430 mμ.

Fig. 4. Protoporphyrin Concentration in Liver.



Comparing the green porphyrin fraction of normal, tumor bearing and toxohormone injected rats, the increase in the 493 mμ-substance was found in the latter two groups as in the case of protoporphyrin fraction. Nevertheless, no noteworthy difference was observed in the absorption at shorter wavelength.

Fig. 5.
a) The absorption curves of "green porphyrin" fraction from normal rat liver (2 cases)

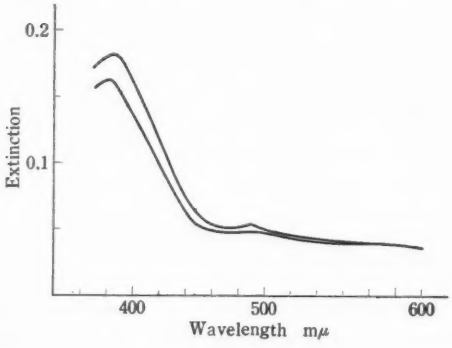


Fig. 5.
b) The absorption curves of "green porphyrin" fraction from liver of rhodamine sarcoma bearing rat (2 cases)

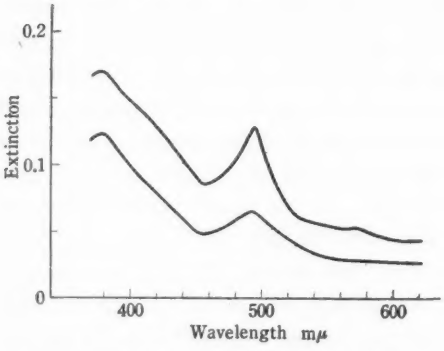
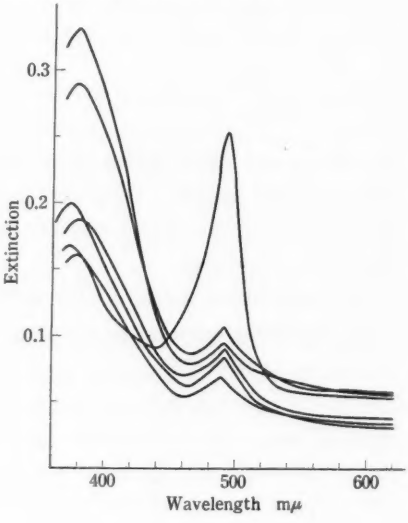


Fig. 5.
c) The absorption curves of "green porphyrin" fraction from liver of Toxohormone injected rat (6 cases)



Protoporphyrin in Harderian Gland

The individual data of the protoporphyrin concentration in Harderian glands are plotted in Fig. 6. In contrast to the rather narrow range of the normal values, i. e., between 10–40 mg/100 g wet weight, the tumor bearing rats showed some extraordinaly high values on one hand, and extremely low ones on the other. This trend of wider dispersion of the values than normal was also detected in the toxohormone injected group.

Coproporphyrin in Urine

An appreciable increase of the coproporphyrin excretion in urine was confirmed in tumor bearing animals, but in toxohormone injected group, no variation of the excretion level in comparison to normal group could be observed. The data are

Fig. 6.
Protoporphyrin concentration in Harderian gland.

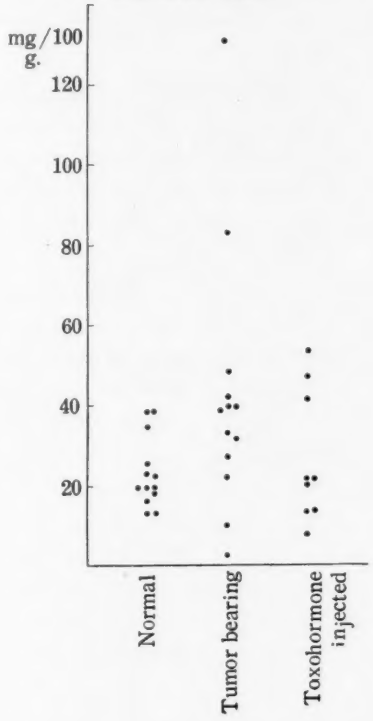
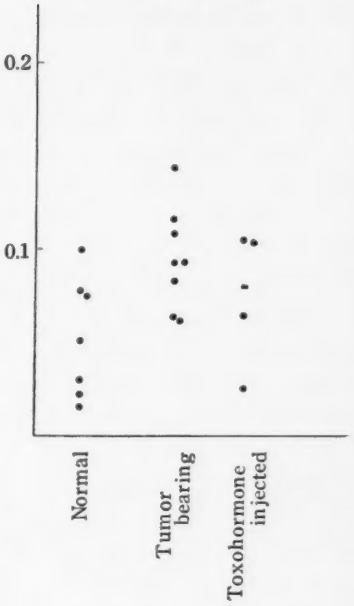


Fig. 7.
Urine coproporphyrin content (extinction at 405 mμ)



illustrated in Fig. 7.

The appearance of 490 mμ absorption material in the coproporphyrin fraction of tumor bearing as well as toxohormone injected rat urine was already pointed out in the experimental section.

Discussion

By the results of the above experiments, it is demonstrated that there is a considerable increase of protoporphyrin contents in liver and coproporphyrin excretion in urine of tumor bearing animals, and that the former trend is also realized by toxohormone injection. The degree of increments observed in these animals is not so striking as in the case of experimental porphyria by Sedormid, therefore these results are more properly to be considered as being due to under-utilization of porphyrin caused by the impairment of iron metabolism, or of the synthesis of protein moieties of hemoproteins, rather than to the disturbance

of porphyrin metabolism itself. This viewpoint may be supported by the fact that no additional or unphysiological porphyrins, not detected in normal rat, are found in the liver or urine of tumor bearing or toxohormone injected rats. Further studies by means of isotopic tracer are hoped to elucidate this situation in unequivocal manner.

The behaviour of protoporphyrin in Harderian gland is very curious, and it is reported that this gland is very sensitive to many factors, e. g., sexual variation, age and nutritional and hormonal conditions (14). It is conceivable that in tumor bearing animals there may be various factors to elevate its level in one stage and to reduce it in another. These may account for the great variation observed in this experiment.

Marver (15) and more recently Begg (16) have pointed out the occurrence of extramedulary hematopoietic tissue in the liver of tumor bearing rat, and the possible danger of making error if one compares the content of chemical substances in liver on the basis of dry weight or nitrogen content between normal and tumor bearing animals. The hematopoietic tissue is supposed to contain porphyrin higher than in liver cells, so it is necessary to examine the possibility that the high concentration observed in this experiment might be due to this reason. Extramedulary hematopoiesis in the liver is neither very constant nor extensive in tumor bearing animals in general. As a matter of fact, by the microscopic examination of the liver of rhodamine sarcoma bearing rat, no such extramedulary hematopoiesis could be detected in any of the cases as far as tested, and so the above possibility pointed out by Begg could be completely set aside in our case.

About the nature of the substance which showed the absorption peak at 493 mμ and was detected in the protoporphyrin and green porphyrin fraction and the coproporphyrin fraction of urine of tumor bearing as well as toxohormone injected rats, no definite conclusion could be made at present. By the position of the absorption maximum, the possibility of it being porphobilin was considered in the first place, which is confirmed as an abnormal product in the intermediate porphyrin biosynthesis and found in some types of porphyria. This possibility was soon rejected because of the very poor solubility of the substance in organic solvent (17). At present time, we are inclined to consider it to belong to urobilin body, since urobilin or urobilinogen has the absorption maximum around 490 mμ and is soluble in organic solvent (17). But even in the cases of liver injury Watson (18) believes that there is no evidence that the liver forms urobilinogen. Thus a satisfactory elucidation of the nature of this substance must await further investigations.

In the preliminary paper, a remarkable increase of free prophyrin in the

erythrocytes of the tumor bearing animals was reported, but that of toxohormone injected ones revealed no significant variation at 24 hrs. after the injection in these experiments. This discrepancy of the behaviours of erythrocyte porphyrin between the two groups may be explained by the fact that the erythrocyte, hemoglobin and erythrocyte catalase have far longer life-spans than that of liver catalase (Theorell (19), Schmid (20)), and actually no decrease of hemoglobin was detected after a single dose of toxohormone. A prolonged treatment with toxohormone in the degree comparable with the case of tumor bearing, may be expected to bring out the increase of erythrocyte porphyrin and decrease of hemoglobin in animals.

Summary

The porphyrin contents in liver, Harderian gland and urine of tumor bearing as well as toxohormone injected rats were examined. A distinct increase of liver protoporphyrin and of coproporphyrin excretion in urine were observed in tumor bearing rats, and the former was also confirmed in toxohormone injected ones.

A substance which showed an absorption peak at 493 mμ was found in the liver protoporphyrin fraction of tumor bearing as well as toxohormone injected rats, and this substance was concentrated in the green porphyrin fraction. Green porphyrin itself did not exhibit a definite variation.

Our hearty thanks are due to Dr. Waro Nakahara for his kind interest and encouragement.

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要　旨

担癌動物のポルフイリン代謝
肝, Harderian 腺, 尿中の遊離ポルフイリン量とこれに及ぼすトキソホルモンの影響

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担癌動物ではヘモ蛋白 (血色素, 肝カタラーゼ等) の合成障害から体内に遊離ポルフイリンが増加すると予想され, 前報ですでに貧血にともなって血球内の遊離ポルフイリンの増加を報告した。その後各臓器内及び尿中排泄ポルフイリン量について検討を加えた。

結果は予想と一致して, 担癌ラッテの肝プロトポルフイリン, 尿中コプロポルフイリンともにかなりの増加を示し, トキソホルモン投与ラッテでも肝プロトポルフイリンの増加を認め得た。Harderian 腺内のプロトポルフイリンは複雑な様相を呈して一定の傾向は得られなかなった。

さらに担癌, トキソホルモン注射ラッテの肝プロトポルフイリン分劃, "Green porphyrin" 分劃さらに尿よりのコプロポルフイリン分劃中には各ポルフイリンの他に 493 mμ で吸光を示す物質が検知されたが, このものの本態はまだ確定に到ってない。

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BIOCHEMICAL INTERACTION OF TRYPAN BLUE AND P-DIMETHYLAMINOAZOBENZENE IN THE LIVER OF RAT (I)

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Although there have been many reports of substances that inhibit carcinogenesis induced by p-dimethylaminoazobenzene (DAB), it has only recently been shown that of these substances, a dry powder of beef liver, yeast, riboflavin and 20-methyl-cholanthrene are most potent as inhibitors. According to E. C. and J. A. Miller (1953), the liver of rat fed with DAB contains aminoazo dyes bound by chemical linkage to certain liver proteins, and the latter two substances, riboflavin and 20-methyl-cholanthrene, decrease the amounts of protein-bound dyes or delay the time required for their reaching the maximum.

It is known that trypan blue is a sulphonated dis-azo dye that somewhat resembles DAB in chemical structure, and recent studies on the biological actions of this vital staining dye, showed the interesting phenomena, reported by Gillman et al., Simpson (1952), and Murakami (1952), whereby the dye injected subcutaneously into rats at weekly or biweekly intervals, induced reticulum cell sarcoma of the liver, while intravenous injections into pregnant mice produced pseudencephalias and other abnormalities of the central nervous system. It has been supposed, on the other hand, that from a microscopic standpoint, trypan blue combines with the reticulo-endothelial cells and parenchymatous cells of the liver, particularly with the Golgi apparatus (Pfuhl, 1932). In connection with these observations, we began a research on the effects of trypan blue on DAB carcinogenesis, with the purpose of studying the biological interaction of trypan blue with DAB in the liver of rat, and as a result confirmed its strong inhibitory effect on liver cancer induction (Iwase and Fujita, 1953, 1954, 1955), (Sayama et al., 1954). It was felt, therefore, that determination of this inhibitory mechanism may provide a fundamental clue to discoveries of similar anticarcinogenic drugs. As a working hypothesis, we considered first whether the protection offered by subcutaneous injections of trypan blue could be explained by the competitive

inhibition of the two dyes; namely, whether DAB competes with trypan blue for the formation of protein-bound dye. However, in some recent studies based on the method of Miller et al., the differences in the amounts of bound DAB occurring between carcinogenic and anticarcinogenic processes were too small for an explanation of the definite inhibitory effect of trypan blue. Furthermore, in our experiments, 1-2 ml. of a 1 per cent aqueous solution of trypan blue (Trypanblau pro injectione, Merck) was injected subcutaneously at intervals of 1-2 weeks, and it was found that it not only induced reticulum cell sarcoma in the liver of rat not fed with DAB, in conformity with the above mentioned bibliography, but also inhibited considerably liver cancer induction by DAB. Hence, the question arises whether the trypan blue used contains simultaneously both anticarcinogen and carcinogen, or there is some agent that induces sarcoma but which inhibits DAB carcinogenesis. Although the subject is under further investigation, at least it is evident as will be demonstrated below, that the product of Merck contains two or three types of dye.

The present paper is concerned with the details of some recent biochemical approaches to these problems, particularly with the biochemical effects of trypan blue injection upon the carcinogenic process induced by DAB.

Methods

The rats were of mixed breed weighing from 80 to 100 g. All experimental animals were divided into the following two groups, i. e., the treated and control groups. The treated group consisted of rats into which 1-2 ml of a 1 per cent aqueous solution of trypan blue was injected subcutaneously at intervals of 1-2 weeks until the total dose of trypan blue for each rat amounted to 60 mg, and these rats were fed with 0.06 per cent DAB containing diets. Animals fed with 0.06 per cent DAB diets and receiving no injection of trypan blue, served as control group I. The basal diet consisted of unpolished rice containing 1.33 mg of riboflavin per kg, and this was supplemented by some green vegetables every two days. DAB feeding was continued until a total does of about 600-700 mg was ingested by each rat. The livers of the treated group at a period of 7 8 months after commencement of the experiment were compared with the controls at the precancerous and cancerous stages for the purpose of a chemical evaluation of the inhibiting effect of trypan blue on liver cancer production. The details of the experimental animals used in this studies are shown in Table I. In almost all cases the gross diagnosis was confirmed by microscopic examination (Iwase, 1954). Comparison was also made with normal livers of rats. Adequate concentrations of isotonic KCl or water homogenates were made from the fresh tissues, using a Potter-Elvehjem homogenizer, and employed for the following experiments.

Table I. Details of the Experimental Animals Subjected to the Chemical Evaluation of the Inhibitory Effect of Trypan Blue.

Experimental group	Trypan blue treatment	Number of rats			Experimental days	Amount of DAB ingested (mg)	Body weight (gm.)		Liver weight (g)	Liver tumour	Tumour incidence (%)
		at initial	at final	Rat No.			at initial	at final			
Treated group	1 ml., at 3, 4, 5, 7, 8, 9, weeks	15	5		154–180	543–592				−	0 (0)
				*	208	670	85	130	9.6	+	
				1	225	675	125	165	9.0	−	
				*	225	643	90	190	21.3	+	
				2	226	669	75	180	9.1	−	
				3	226	712	105	190	13.3	−	
				*	231	656	80	115	12.5	+	
				4	233	692	85	205	9.1	−	
				5	236	641	95	170	6.5	−	
				6	236	665	100	215	10.5	−	3 (33)
Control group I		10	10	1	163	646	80	160	30.3	+	
				*	163	663	85	160	23.5	+	
				2	164	681	85	175	17.3	+	
				3	164	652	60	135	33.5	+	
				4	167	653	80	140	18.9	+	
				5	167	670	85	210	11.0	+	
				*	182	709	85	130	19.5	+	
				6	190	731	85	180	18.0	+	
				7	197	732	85	155	39.3	+	
				8	199	681	80	165	50.1	+	10 (100)

* These rats died before being sacrificed. A rat that died within 100 days was omitted. The data of Table I has been referred to from the report of Iwase (1954), and hence, the details of gross findings and histology were omitted.

Assay of Four Oxidases—The enzyme activities were determined manometrically, for succinic dehydrogenase, cytochrome oxidase, d-amino acid oxidase and xanthine oxidase. The reaction mixtures were made according to Schneider, and Potter (1943), Ogihara and Tyuma, (1949), and Axelrod, and Elvehjem (1941). The centre well of each flask contained 0.2 ml of 2N KOH. After 5 minutes equilibration in the bath at 38°C., the taps were closed, and the oxygen uptake was read. Among the reaction mixtures used was cytochrome C prepared from beef hearts according to the procedure described by Keilin and Hartree, with the modification that the enzyme was dialyzed against glass distilled water instead of 1 per cent NaCl solution. Flavin adenine dinucleotide was prepared from pig-liver, according to the procedure reported by Crammer (1948).

Estimation of Catalase Activities and Flavin Compounds—Catalase activity was determined according to the method described by Euler and Josephson (1927). Riboflavin and its derivatives, i. e., flavin adenine dinucleotide and flavin mononucleotide were measured by the following fluorometric determinations based on the lumiflavin method described by Kuhn et al. After placing pieces of fresh tissue into a water bath at 80°C. for 5 minutes, adequate concentrations of water homogenate were made using a Potter-Elvehjem homogenizer. By keeping the homogenate at 80°C. for 15 minutes, flavin compounds were extracted in water, and to an aliquot of the extract the same volume of 1 N NaOH was added, and was then exposed to a 200 W. electric lamp for 1 hour to convert the flavin compounds into lumiflavin. The corresponding concentrations of lumiflavin formed could be extracted by chloroform when the medium was acidified by adding acetic acid. The fluorescense of the chloroform layer was compared with the standard lumiflavin chloroform solution made from standard riboflavin solution, using the Klett fluorometer. The values obtained were regarded as total riboflavin contents. Further, the flavin compounds contained in the water extract were condensed according to the procedure described by Crammer and were then divided into the three forms of flavin compounds by filter paper chromatography in which as developer a 4 : 1 : 5 n-butanol-acetic acid-water mixture was used (Yagi, 1951).

Nucleic Acid Analysis—Homogenates of the fresh livers were analyzed for ribonucleic acid phosphorus and desoxyribonucleic acid phosphorus by a modification of the methods described by Schneider, and Schmidt and Thannhauser. Namely, after removal of the "acid soluble" phosphorus and the lipoid phosphorus from the samples according to the procedure described by Schneider (1945), the residues were analyzed by the Schmidt and Thannhauser method (1945).

Here too, a subsequent biological experiment was made with the purpose of studying the protein-deletion hypothesis suggested by Miller and his coworkes, and also the influence of trypan blue upon the early stage of DAB carcinogenesis.

All experimental animals were divided into the following three groups, i. e. the treated group and the control group I as described previously, and a control group II. The control group II consisted of animals which were injected with trypan blue but received no DAB. Unfortunately in this series of experiment many of the animals fed with DAB were in poor condition, and died within 30 to 60 days with congestion of the lung. However, feeding was continued until the animals either died or were sacrificed at the ends of the experimental periods of 2, 4 and 6 weeks. Three rats in which the general appearance was comparatively good were selected at each period after commencement of experiment, and sacrificed for estimation of bound DAB, distribution of each dye in the cellular components of the liver, and for others.

Preparation of Crude Liver Precipitates, and Estimation of Bound DAB— For this experiment the method described by Miller and his coworker (1947) was used routinely, but in order to facilitate the discussion later, the procedure will be described in detail. The perfused liver of rat was minced, and 20 per cent water homogenates were prepared. To the homogenate the same volume of 1 M sodium acetate buffer, pH 5, was added, and boiled for 3 minutes. After centrifugation the protein coagulum was washed once with 20 ml of acetate buffer, and twice with 20 ml. portions of 95 per cent ethanol. The rest of the protein contained in the supernatant solution or acetate washing was precipitated by the addition of trichloracetic acid. The wet precipitates, wrapped individually in quantitative filter paper, were extracted at approximately 60°C. with 95 per cent ethanol in a Soxlet extractor for 48 hours to remove free aminoazo dyes and lipids. They were then transferred to small beakers, dried to light powders over sulfuric acid in vacuo, and stored in tightly closed vials. The dry powders represented 15.6 to 25.6 per cent (av.=20.1) by weight of the original fresh liver. 50 mg samples of powder were digested in a mixture of 2 ml of ethanol and 5 ml of 4.5 N KOH for 20 hours in 25×200 mm pyrex test tubes equipped with a finger condenser. The tubes were heated for 20 hours in a water bath at 80°C. to which a layer of liquid paraffin was added to prevent heat loss. After digestion the samples were cooled, and 2 ml. of ethanol, 4 ml. of 11 N KOH, and 10 ml of peroxide-free ethyl ether were added to each tube. Then the digests were stirred for 5 minutes with a capillary pipette operated by air pressure as strongly as possible. The ether layers were transferred to clean Thunberg tubes, and the aqueous residues were then re-extracted with 6 ml of a 1 : 5 ethanol-ethyl ether mixture as described above. The combined ether layers were evaporated to about 5 ml in a water bath at atmospheric pressure and then brought to near-dryness (0.1 ml) in vacuo. The residues were dissolved in 2 ml of ethanol delivered evenly over the inside of the tubes and 2.5 ml of 7 N HCl were added to develop the colours. 1 ml of

light petroleum ether was added to each tube, and the tubes were shaken to cause insoluble residue if any, to collect at the interface. After centrifugation, the samples were then poured into matched 10×10 mm pyrex tubes, and read in a Beckmann DU spectrophotometer at 520 mμ, 15 to 30 minutes after the addition of the acid. All values were expressed as E (log $I_0/I)_{520\,m\mu}$ per 100 mg dry powder under the conditions described.

Enzyme Hydrolysis of the Crude Protein Powder—The procedure for prolonged tryptic hydrolysis of the dry powder was the same as that described by Miller et al. (1947). Degradation of the powder with acid and alkaline nucleotidase was performed according to the method described by Contardi and Rovazoni (1935) and Klein (1932). Each enzyme was prepared by the procedures described by the above authorities.

Electrophoretic analyses—Electrophoretic analyses were carried out on the soluble liver proteins of rats injected trypan blue. The procedure for schlieren-scanning method was the same as that for soluble liver proteins of rats fed with DAB, reported by Hoffmann and Schechtman (1952), using a "Shimazu" electrophoresis apparatus. Paper electrophoresis was carried out on an aqueous solution of trypan blue, and some related dyes, using the following two buffers, i. e., veronal buffer (pH 8.3, ionic strength-0.06) and phosphate buffer (pH 5.6, ionic strengh-0.056).

Comparison of Trypan Blue Contents between the So-called Total and Insoluble Proteins—Five rats were sacrificed at a period of 1 week after the subcutaneous injection of 1 ml of a 1 per cent trypan blue salution. 1 ml of 20 per cent water homogenates of the livers were subjected to the nucleic acid determination by the method described by Schneider. The levels of trypan blue contained in his fourth fraction, viz. the protein residue, were expressed arbitrarily in terms of E (log 10 $I_0/I)_{590\,m\mu}$ per the total volumes of 5.0 ml, and regarded as the amounts bound to the total proteins. The rests of the homogenates were dialyzed, and corresponding same volume was ultracentrifuged, according to the procedure described by Hoffmann and Schechtman. The levels of the dye contained in the fourth fraction of the precipitates were determined by the same method, and the results obtained were regarded as the amounts bound to the insoluble proteins.

Application of Paper Chromatography and Chromatography on Aluminium Oxide Column for Trypan Blue—The aqueous solutions of trypan blue, and of H-acid were separated by paper chromatography (in which as developers were used the upper layer of a 4 : 1 : 2 ethyl acetate-ethanol-water mixture, and the upper and lower layers of a 4 : 1 : 5 n-butanol-ethanol-water mixture), and by adsorpto-chromatography on a 3×15 cm column of aluminium oxide (standerdisiert

Al_2O_3 zur chromatographischen Adsorptionsanalyse nach Brockmann).

Results

Inhibitory Effects of Trypan Blue on DAB Carcinogenesis

Just as certain tissues possess morphologically certain characteristics, so the quantitative distribution of various enzymes contained in these tissues are found to exhibit patterns peculiar to the tissues (Greenstein, 1947). Hence, in liver subjected simultaneously to ingestions of DAB and injections of trypan blue, at a stage when in the control rat there is already seen development of DAB cancer, it will not be in vain to evaluate biochemically the anticarcinogenic powers of trypan blue by investigating whether the metabolism of the liver is in cancerous or precancerous stage, or normal. Regarding the biochemical differences seen in normal liver and DAB induced cancer, there have been systematic and detailed reports by Greenstein and coworkers, but in this paper, based on the recent

Table II. Enzyme and Coenzyme Assays on the Homogenates from Livers of the Treated Group.

	Rat No.	Normal liver	Liver cancer	Livers of the treated group
Succinic dehydrogenase*	1	49.4	9.96	55.6
	2	35.7	8.12	31.62
	3	—	—	60.6
Cytochrome oxidase*	1	305.0	75.0	289.0
	2	297.5	46.0	179.0
	3	—	—	318.0
D-amino acid oxidase**	1	11.10	0.80	5.57
	2	11.87	1.59	3.48
	3	—	0.95	—
Xanthine oxidase**	1	15.65	4.45	6.30
	2	14.00	4.23	8.20
	3	—	4.58	—
Flavin adenine dinucleotide***	1	29.36	6.57	30.73
	2	27.84	9.85	31.47
	3	—	8.77	—

* Data expressed in terms of cmm. oxygen taken up in 10 minutes by 20 mg fresh weignt of tissue.

** Data expressed in terms of cmm. oxygen taken up in 10 minutes by 50 mg fresh weight of tissue.

*** Data expressed in terms of microgram per 1 g fresh weight of tissue.

work of Potter and his coworkers, Masayana and Yokoyama, Davidson and Waymouth, and Schneider, Westerfeld and his coworkers (Miller, 1953), Kensler, and Sugiura, Nakahare and Fukuoka, that concerned especially with succinic dehydrogenase, cytochrome oxidase, d-amino acid oxidase, xanthine oxidase, nucleic acids, riboflavin and its derivatives, and catalase were studied, with the livers of the treated group, and normal livers, DAB-induced precancerous and cancerous livers of rats as materials. Table II shows the results thus obtained with succinic dehydrogenase, cytochrome oxidase, d-amino acid oxidase, xanthine oxidase and flavin adenine dinucleotide.

It may be noted in Table II that the concentrations of succinic dehydrogenase and cytochrome oxidase in normal livers and tumours are lower than the values observed by other authorities. Such results may be due to the fact that the

Table III. Distribution of Three Forms of Riboflavin in Normal Liver, Liver Cancer, and Liver of the Treated Group.

	Rat No.	Total Riboflavin	Flavin Adenine Dinucleotide		Flavin Mononucleotide		Free Riboflavin	
				% *		% *		% *
Normal liver	1	25.38	24.95	98.30	0.38	1.49	0.05	0.21
	2	29.61	29.08	98.21	0.44	1.48	0.09	0.31
	3	30.85	29.36	96.36	0.96	3.11	0.16	0.26
	4	30.33	27.84	91.79	2.34	7.71	0.15	1.50
	Average	29.04	27.80	96.16	1.03	3.45	0.11	0.57
Liver cancer	1	3.61	3.49	96.67	0.11	3.04	0.01	0.29
	2	2.87	2.22	77.35	0.64	22.29	0.01	0.36
	3	5.85	3.93	67.40	1.88	32.24	0.02	0.36
	4	6.99	6.57	93.99	0.40	5.72	0.02	0.29
	5	10.42	9.85	94.52	0.52	5.18	0.03	0.30
	6	11.39	10.99	96.48	0.37	3.24	0.03	0.28
	Average	6.85	6.17	87.73	0.65	11.95	0.02	0.33
Liver of the treated group	1	35.90	35.15	97.91	0.72	2.01	0.03	0.08
	2	30.79	26.85	87.20	3.35	9.98	0.52	2.82
	3	27.55	26.20	95.09	1.27	4.61	0.08	0.30
	4	32.35	30.73	94.99	1.46	4.51	0.16	0.50
	5	33.03	31.47	95.27	1.48	4.48	0.08	0.25
	6	39.41	38.46	97.58	0.87	2.21	0.09	0.11
	Average	33.32	31.47	94.67	1.52	4.61	0.17	0.69

* Values for the total riboflavin contents were taken as 100.
All other values were expressed in terms of microgram per 1 g fresh weight of tissue.

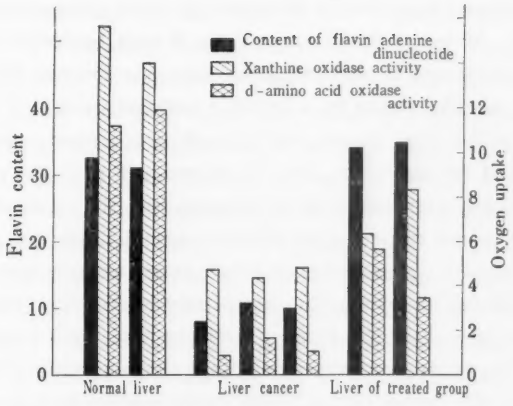


Fig. 1. The contents of flavin adenine dinucleotide are illustrated in terms of microgram per 1 g wet weight of tissue. Two oxidase activities are shown in terms of cmm of oxygen absorbed per 50 mg wet weight of each tissue per 10 minutes.

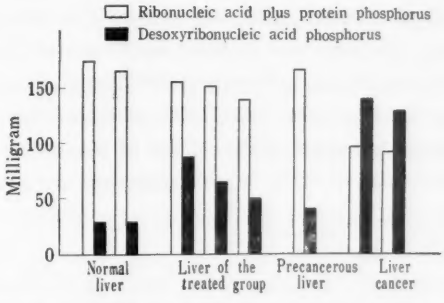


Fig. 2. The contents of desoxyribonucleic acid phosphorus and ribonucleic acid plus protein phosphorus are illustrated in terms of mg per 100 g fresh tissue.

activities of the succinic dehydrogenase in the liver changed when the diet was altered (Potter et al., 1950). However, it is apparent that the concentrations of succinic dehydrogenase and cytochrome oxidase in the livers of the treated group are significantly higher than the values observed in tumours that occurred 5–7 months after the biginning of 0.06 per cent DAB administration. The data shown in Figure 1 are based on that for yellow enzymes and their coenzyme shown in Table II. As may be seen in this figure, the values of d-amino acid oxidase and xanthine oxidase observed in the livers of the treated group were either in between or at near the lower extreme of the range for the livers of the other two groups, but the contents of flavin adenine dinucleotide which is their coenzyme were not significantly different from that for normal liver. The differences in the

levels of flavin adenine dinucleotide between the three groups are given in greater detail in Table III. Based on the above results, it may be considered that regarding the enzymes subjected to determination here, the liver of the treated group at a period of 7-8 months shows as a whole a metabolism which was significantly different from that for liver cancer, and though not perfect, seemed in general to resemble that of normal liver. But as shown in Fig. 2, the results of nucleic acids analyses did not necessarily point to such, and the content of desoxyribonucleic acid was found to be higher than in normal liver, though lower than in liver tumour. Recently, in preliminary experiments we reported that subcutaneous injections of 80 mg of trypan blue completely inhibit the formation of liver cancer up to 7 months after the start of 0.06 per cent DAB feeding, but in the later months of the experiment there results a characteristic proliferation of the histiocytic cells in the portal tracts. The livers subjected to determination here, were ones obtained 7-8 months after start of experiment, hence the reason for the high content of desoxyribonucleic acid may be due to in part to increase in cellular components, especially to a marked histiocytic response.

Of the many chemical changes that accompany the development of azo dye cancer, those involving riboflavin and catalase have especially received widespread investigation. Kensler and his coworkers, and Griffin and Baumann have reported that the riboflavin content of the liver decreased in rats that have been fed with certain carcinogenic azo dyes, while Miller et al. indicated that the rats and extent of the riboflavin loss of liver fed with various azo dyes are influenced in

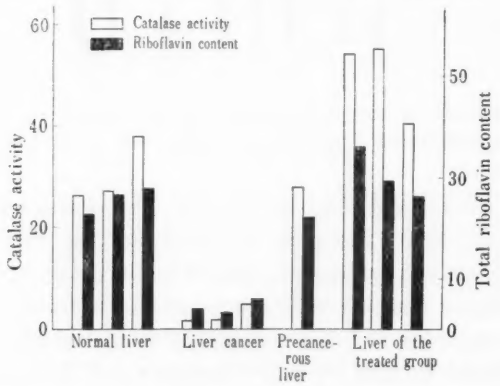


Fig. 3. Catalase activity is expressed in terms of $\frac{1}{T} \ln \frac{A}{A-X}$ per 1 g wet weight. Minutes for digestion are expressed as T; titration value of 5 ml of the digest at 0 time in terms of 0.005 N permanganate as A; and the titration value after T minutes as A-X. Total riboflavin contents are showed in terms of microgram per 1 g wet weight of tissue.

proportion to the carcinogenic activities of the respective azo dyes (Kensler et al., 1940, Griffin and Baumann, 1946, Miller et al., 1948). Similar observations have been reported by Nakahara et al. and many other investigators regarding catalase activity, and it has been found that when rats are given DAB, the catalase activity of the liver decreases gradually with the lapse of days (Miyaji, 1948). As is shown in Fig. 3, the data obtained by us also showed a similar tendency, and the riboflavin and catalase contents of the liver became lowred in the order of cancer, precancer when compared with normal liver. The values for the livers of the treated group were the same or slightly higher than those for normal liver. It was found further that depending on the individual, the increase and decrease in riboflavin and catalase present parallel relationships. In other words, when the content of riboflavin is high or low in the liver, there are seen corresponding high or low values for the activities of catalase.

What roles the many changes existing between normal and primary cancerous tissues reported by numerous investigators play in the carcinogenesis remain almost unclarified. However, concerning at least liver cancer, there remains no doubt regarding the existing view that the metabolism in cancerous liver differs from that in normal liver. Hence, when we consider the results of chemical changes described in this paper, the efficacy of subcutaneous injections of trypan blue as an anticarcinogen may be considered generally, to be very high, though there was seen some increase in cellular components, seen morphologically and as the result of nucleic acids analyses.

Nature of the Anticarcinogenic Activity Presented by Subcutaneous Injections of Trypan Blue.

Interesting unique findings have been reported by Miller et al. regarding the nature of DAB carcinogenesis and the inhibitory mechanism due to the administration of riboflavin and 20-methyl-cholanthrene. In order to clarify the biochemical nature of the liver cancer inhibition presented by trypan blue, we investigated first in what state trypan blue exists in the liver of rat. As a preliminary step trypan blue was injected at weekly intervals, and the perfused livers of rats were subjected to nucleic acid determination by the method described by Schmidt and Thannhauser. The results showed that almost all the trypan blue in the liver are contained chiefly in the protein plus nucleoprotein residue, viz. the third fraction of Schmidt and Thannhauser (Iwase, and Fujita, 1953). Further, as shown in Table IV, the trypan blue attached to the hepatic cell components is not removed by treatment with hot trichloracetic acid (based on the method of Schneider), but is collected in the protein residue, viz. the fourth fraction of Schneider's method. From control groups I and II, and from the treated group, 20 per cent homogenates of the liver were prepared, and after the

Table IV. A. The Levels of Trypan Blue Contained in Schneider's Four Fractions Which were obtained from Rat Livers of the Three Groups.

	Control group I*	Control group II**	Treated group***
Rat No.	23	26	25
1 st. Fraction	0.017	0.034	0.018
2 nd. Fraction	0.003	0.022	0.002
3 rd. Fraction	0.010	0.035	0.060
4 th. Fraction	0.093	0.695	0.424

* One rat received p-dimethylaminoazobenzene continuously in diets for 6 weeks.

** One rat received 5 injections of 10 mg trypan blue, but no ingestion of p-dimethylaminoazobenzene for 6 weeks.

*** One rat received 5 injections of 10 mg trypan blue and continuous feeding of p-dimethylaminoazobenzene containing diets for 6 weeks.

1 ml. of 20 per cent homogenates prepared from each fresh tissue were subjected to Schneider's method. All values were expressed arbitrarily as E (log Io/I) $_{590\ m\mu}$ per 5 ml under the conditions described.

B. The Levels of Extinction Coefficients (at 590 mμ.) in the Schneider's 4 th. Fraction extracted from Rat Livers at Periods of 2 and 6 weeks.

Experimental group	Treatment	Rat No.	At a period of "2" weeks*	At a period of "6" weeks**
Control group I	0.06 % p-dimethylaminoazobenzene (DAB) feeding	13	0.144	—
		14	0.130	—
		15	0.106	—
		20	—	0.077
		21	—	0.170
		23	—	0.093
Control group II	1 injection of 10 mg trypan blue	16	0.460	—
		17	0.421	—
		18	0.756	—
	5 injections of 10 mg trypan blue	22	—	0.702
		24	—	0.372
		26	—	0.695
Treated group	1 injection of 10 mg trypan blue and DAB feeding 5 injections of 10 mg trypan blue and DAB feeding	10	0.627	—
		11	0.530	—
		12	0.424	—
		19	—	0.401
		25	—	0.424
		27	—	—

*, ** Each period corresponds to that after the start of DAB administration. All values were determined, and expressed under the conditions similar to the above.

"acid soluble" substances, lipoids and "hot trichloracetic acid soluble" substances were removed, the residue was dissolved by heating for 15 minutes at 80°C in 0.2 per cent NaOH solution. The absorption spectra of the solutions

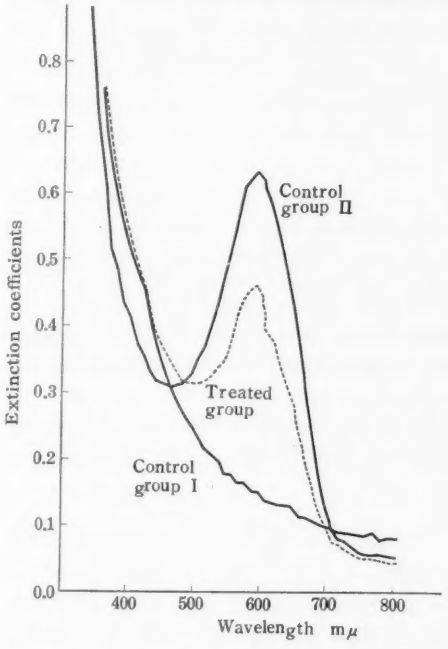


Fig. 4. Absorption spectra of bound dyes present in the 4th. fraction of Schneider's method. From No. 9 of control group I and II, and from No. 8 of the treated group, 20 per cent homogenates of the livers were prepared, and 1 ml. of each homogenates subjected to Schneider's method was determined in his 4th. fraction under the conditions described.

were determined in matched 10×10 mm. pyrex tubes, using a Beckmann DU spectrophotometer, and the results are shown in Fig. 4. The shapes of the 3 curves thus obtained, also suggest that trypan blue, similar to DAB, is either attached to or bound to the hepatic cell components, probably the proteins. Again, the fact that the trypan blue attached is not soluble in hot trichloracetic acid, indicates that the trypan blue injected is either not bound to the desoxyribonucleic acid and ribonucleic acid of the liver cells, or that the dye-nucleic acid compounds are not extractable by hot trichloracetic acid. Hence, crude liver protein precipitates were prepared by the method of Miller et al. from each of the experimented groups, and the powders were hydrolyzed with acid and alkaline nucleotidase; but the trypan blue as well as DAB, was not liberated (Table V).

The recent studies of Price et al. (1948) on the distribution of protein-bound DAB in the liver cells of the rat, are especially of interest. According to them, when by the differential centrifugation technique, liver homogenate was divided into four fractions, of nuclei, large granules, small granules and supernatant, the concentration of protein-bound DAB was found to be highest in the small

Table V. Degradation of Crude Liver Protein Precipitates with Trypsin and Nucleotidases.

Enzyme	Levels of the dye which could be extracted from the digests with ethanol-ethyl ether.*		
	Control graup I	Control group II	Treated group
Trypsin	0.010	0.054	0.075
Acid nucleotidase	0.024	0.015	0.008
Alkaline nucleotidase	0.036	0.000	0.015

For these experiments 100 mg samples ot the crude protein powders prepared at a period of 6 weeks after the start of p-dimethylaminoazobenzene feeding were used. The data indicate the maximum values of results obtained from 2-3 estimations.

* The levels of p-dimethylaminoazobenzene which could be extracted from the digests were expressed in terms ot E $(\log_{10} I_0/I)_{520}m\mu$. of acid-ethanol solutions described under Estimations of the bound dye.

The levels of trypan blue contained in the digests were determined in terms of E $(\log_{10} I_0/I)_{590}m\mu$. but only negligible amounts of dye were obtained from the control group II and treated group.

granules and supernatant fractions. As we did not have a similar apparatus available, Dounce's we employed the Dounce's method (1943) to determine the distribution of subcutaneously injected trypan blue within the cellular constituents of the liver. The results which were obtained from the livers of rats at a period of 7 days after 1 injection of 10 mg trypan blue showed that with progress of the preparation, the nuclear sediment begins to loss the blue colour indicative of the presence of trypan blue, till in the final stage it assumed a pure white rubber like appearance. Further when nucleohiston was prepared from the same liver according to the method of Mirsky, and Pollister (1942), such preparation contained no dye stuff. These findings may support the early observations of Bowen and Kedrowsky, that the trypan blue in the cells is bound to cellular components other than the nucleus, (probably to the protein of Golgi's apparatus).

The above results obtained from preliminary investigations agree with the corresponding series of experimental results reported by Miller and coworkers on DAB, and it is naturally suggested that the definite anticarcinogenic effects of trypan blue seem to be based on the conflict between trypan blue and DAB against the formation of protein-bound dye. Hence, a similar biological experiment was freshly undertaken, and the livers obtained were used for determinations of protein-bound DAB. It has already been reported by E. C. and J. A. Miller (1947) that when rats are fed with DAB the amount of protein-bound dye in the liver

reaches a maximum between the 4 th. and 6 th. weeks after commencement of the feed, followed by a gradual decrease later. Also, they pointed out that the speed for the bound dye to attain a maximum depends directly upon the carcinogenic power of some dyes concerned. In the present series of experiments, due to the high mortality of the experimented animals, the feeding with DAB was discontinued after 6 weeks. But it was considered that results compared at the ends of 2 nd, 4 th and 6 th weeks, would be sufficient for a conclusion to be made, concerning the competition of the two dyes. As shown in Fig. 5, during the 6 weeks of DAB feeding the amount of protein-bound DAB in the livers of the treated group was either approximately equal to or slightly higher or lower than that of livers in the control group I. The result given in Fig. 5 is based on the averages of data that are given in greater detail in Table VI. Further as may be noted from Table V, the amounts of DAB liberated by prolonged tryptic hydrolysis were found to be even slightly greater in the livers of the treated group than that of control group I. From these results it became clear that on the basis of the method of Miller et al., the differences in the amounts of bound DAB between the treated and control groups are too small and an explanation for the marked inhibitory effects of trypan blue cannot be made from a hypothesis of conflict of the two dyes. However, as was stated before, the precipitates of liver protein used for determinations of protein-bound DAB were crude preparation. Hence, only when the differences in the amounts of bound DAB between the treated and control groups become fairly large, a working hypothesis that the two dyes are competing with each other in the formation of protein-bound dye, can be made with comparative safety. Even though no differences are demonstrable when crude preparations are used, a significant difference may be demonstrable when the amounts of DAB bound to some particular protein of the crude preparations are compared. It therefore becomes necessary first to determine whether the protein or proteins to which the trypan blue is attached or bound are similar to or not to the protein bound to DAB. To clarify this problem the livers of control group II were subjected to electrophoretic studies. It has already been made clear by Sorof et al. (1951) that over 50 per cent of the bound dye in the livers of rats fed with DAB are bound to the soluble proteins, and that 70–90 per cent of this are contained in the slow-moving proteins, which account for only 7–15 per cent of the total soluble proteins (Miller, 1953). Based on the method of Hoffmann and Schechtman, and using a Shimadzu electrophoresis apparatus, the pattern resulting was photographed on monochromatic and colour films. However, contrary to the results of the above authorities on DAB, we failed to obtain an evidence that the trypan blue present in the liver of control group II are bound especially to the slow-moving

Table VI. Details of the Estimation of Bound DAB at Periods of 2, 4 and 6 Weeks after Commencement of DAB Feeding.

Experimental Results

Group	Rat No.	1st. Estimation	2nd. Estimation	3rd. Estimation	Average for an Individual	Average for Group
		At a Period of "2" Weeks				
Control Group I #	13	0.134	0.128	0.102	0.121	0.160
	14	0.172	0.172	0.170	0.171	
	15	0.208	0.184	0.176	0.189	
Treated Group ##	10	0.122	0.150	0.124	0.132	0.134
	11	0.112	0.144	0.158	0.138	
	12	0.150	0.112	0.132	0.131	
Control Group II ###	16	0.056	0.052	—	0.054	0.050
	17	0.064	0.040	0.064	0.056	
	18	0.040	0.052	0.026	0.039	
		At a Period of "4" Weeks				
Control Group I #	2	0.206	0.130	—	0.168	0.201
	4	0.250	0.297	—	0.274	
	8	0.154	0.154	0.176	0.161	
Treated Group ##	1	0.174	0.172	—	0.173	0.184
	6	0.192	0.184	—	0.188	
	9	0.216	0.190	0.168	0.191	
Control Group II ###	3	0.044	0.030	0.024	0.033	0.034
	4	0.044	0.030	0.044	0.039	
	5	0.024	0.032	0.030	0.029	
		At a Period of "6" Weeks				
Control Group I #	20	0.280	0.286	0.230	0.265	0.259
	21	0.238	0.330	0.206	0.258	
	23	0.308	0.268	0.190	0.255	
Treated Group ##	19	0.330	0.320	0.234	0.295	0.267
	25	0.270	0.240	0.204	0.238	
	27*	—	—	—	—	
Control Group II ###	22	0.050	0.040	0.052	0.047	0.060
	24	0.080	—	—	0.080	
	26	0.054	—	—	0.054	

* This rat died with pneumonia one day before sacrifice.

\# These rats received p-dimethylaminoazobenzene continuously in the diets for 2, 4 and 6 weeks.

\## These rats received continuous ingestions of p-dimethylaminoazobenzene diets, and 1, 3 and 5 injections of 10 mg trypan blue for 2, 4 and 6 weeks. respectively.

\### These rats received 1, 3 and 5 injections of 10 mg trypan blue for 2, 4 and 6 weeks respectively, but no ingestion of p-dimethylaminoazobenzene.

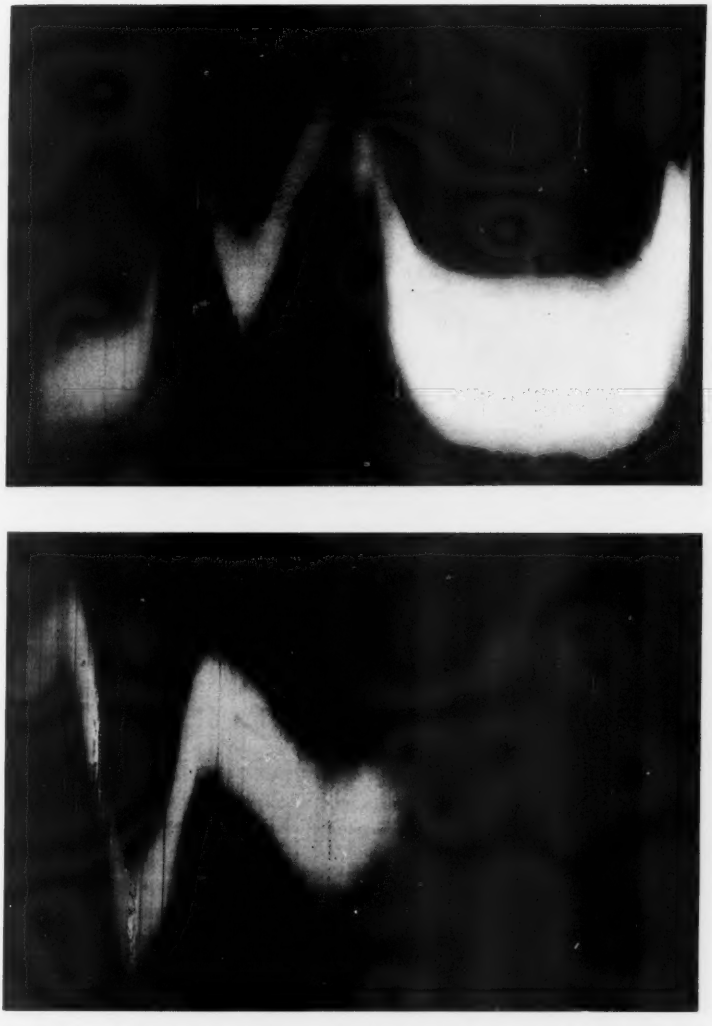


Fig. 6. The electrophoretic patterns of the soluble liver proteins of rats at a period of 1 week after 1 injection of 10 mg trypan blue.

The electrophoretic analyses indicated were conducted in veronal buffer (pH 8.45, 0.144 μ) with a 2 ml cell at 150 V, 14.5 mA for 60–80 minutes, using a Shimadzu electrophoresis apparatus.

Upper photograph—Ascending pattern

Lower photograph—Descending pattern

The colours of these patterns were referred to from the results of the corresponding colour prints.

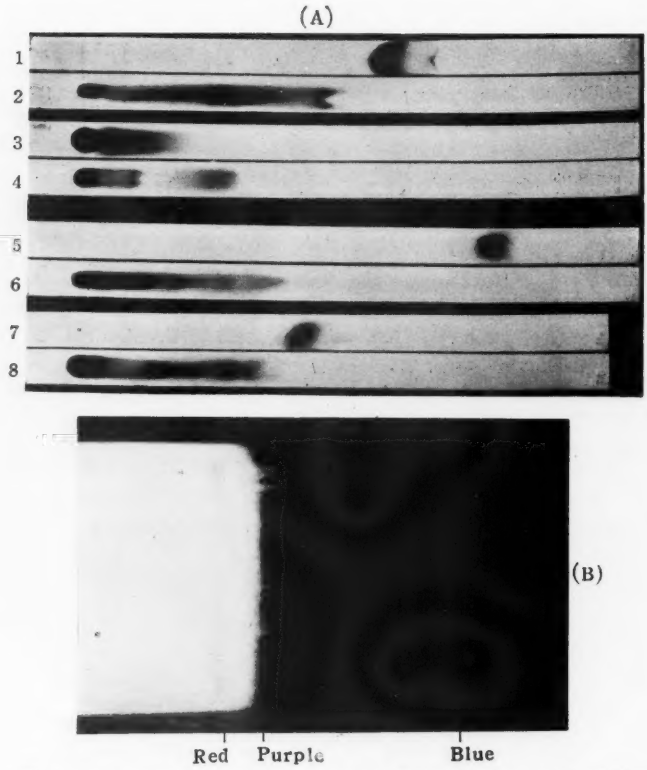


Fig. 7. The paper chromatogram and adsorpto-chromatogram on aluminium oxide.

(A) The paper chromatogram

Strip No.	Material	Developer
1	H-acid	Lower layer of 4 : 1 : 5 n-butanol-ethanol-water
2	Trypan blue	
3	H-acid	Lower layer of 4 : 1 : 2 ethyl acetate-ethanol-water
4	Trypan blue	
5	H-acid	Upper layer of 4 : 1 : 5 n-butanol-ethanol-water
6	Trypan blue	
7	H-acid	Upper layer of 4 : 1 : 2 ethyl acetate-ethanol-water
8	Trypan blue	

For these experiments 0.05 ml of 1 per cent aqueous solutions of trypan blue and of H-acid were applied.

(B) The adsorpto-chromatogram on aluminium oxide

20 ml of a 1 per cent aqeous solution of trypan blue (Merck) were separated by adsorpto-chromatography on aluminium oxide column (in which as a developer was used water) into two or three types of dye.

The colours indicated in these photographs were referred to from the corresponding colour prints.

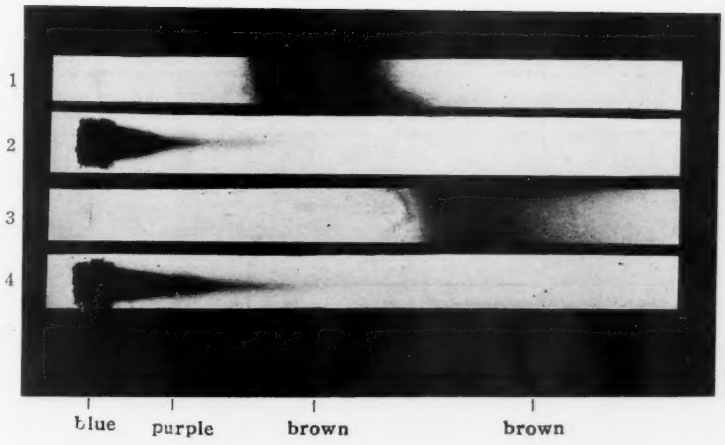


Fig. 8.—The paper electrophoretic analyses of 1 per cent aqueous solutions of trypan blue and of H-acid.

The electrophoretic analyses of 0.02 ml samples were conducted in veronal buffer (pH 8.5, 0.06 Mol) and phosphate buffer (pH 5.6, 0.05 Mol) at 600 V., 1.5 mA. for 6 hours.

Strip No.	Material	Buffer
1	H-acid	0.05 Mol phosphate buffer (pH 5.6)
2	Trypan blue	
3	H-acid	0.06 Mol veronal buffer (pH 8.5)
4	Trypan blue	

proteins of the soluble proteins (Fig. 6). Of course in the case of photographs on colour films, the colour temperature was adjusted as much as possible to that suitable for the film, and the exposure extended to the maximum possible for the particular setup. When the results obtained were considered, it appeared necessary that with this apparatus attention should be paid to the optical system being not apochromatic throughout the entire field of visible rays, and that exposure time was not sufficient. Hence, we are at present devising a new apparatus to cope with these defects, as well as an electrophoresis-convection apparatus (Cann and Kirkwood et al., 1949); the results obtained with these will be reported in the near future. The paper electrophoresis that we have been using so far has also not produced clearcut results. Also, during the preliminary stage of electrophoretic analysis, it was found that the attached or bound trypan blue did not pass through the dialysable membrane, and that a fair amount of the dye remained in the insoluble precipitate obtained by ultracentrifugation (15000 r.p.m.) for 30 minutes. In view of these, a determination was made to find out what percentage of the total attached or bound trypan blue was contained in the

Table VII. Comparison of Trypan Blue Contents in the So-called Total and Insoluble Proteins.

Rat No.	Trypan blue contents in total proteins	Trypan blue contents in insoluble proteins
1	0.2616	0.0585
2	0.2666	0.0365
3	0.1746	0.0665
4	0.2206	0.0265
5	0.2006	0.0765
Average	0.2248	0.0529

1 ml of 20 per cent liver homogenates were prepared from the rats at a period of 1 week after the subcutaneous injection of 1 ml of a 1 per cent trypan blue solution. The levels of trypan blue contained in Schneider's fourth fraction, viz. the protein residue, were expressed arbitrilly in terms of E ($\log_{10}$ I_0/I) 590 mμ per total volume of 5.0 ml under the conditions described, and regarded as the amounts bound to the total proteins. The corresponding volumes of the homogenates were dialyzed, and ultracentrituged, according to the procedure described by Hoffman, and Schechtman. The precipitates were determined by the same method, and the results obtained were regarded as the amounts bound to the insoluble proteins. All E values were corrected by that for normal liver.

insoluble precipitate of rat liver. The results shown in Table VII, indicated it to be about 23.6 per cent.

Next, besides the above-mentioned experiments based on the protein deletion hypothesis of Miller, and his coworkers, an examination was made to determine the purity of the trypan blue (Merck preparation), in order to consider the two different activities, carcinogenic and anticarcinogenic, that the dye presents for malignant tumours. Recently, Simpson (1952) reported that when trypan blue is used to induce reticulum cell sarcoma of rats, trypan blue B. P. C. (Avlon brand, I. C. I.) when compared with Gruebler's products, showed practically little ill effect, and the lymphnode tumours and some of the other liver lesions were not observed; but there were seen almost no considerable differences in the carcinogenic activities obtained with the reticulum cell sarcomas. The Merck product of trypan blue used by us resembled the Gruebler's product in that there resulted loss of weight and appetite, and a fairly high mortality. As has been pointed out by Simpson, and colour index of Merck, the commercial brands are not pure but contain a variety of impurities, and in our experiments too, this fact was substantiated by the results of paper chromatography, adsorpto-chromatography on aluminium oxide and paper electrophoresis (Fig. 7 and 8). Of the variety of impurities, one or the other is related to the toxicity of trypan blue, and the differences in toxicity of the various brands of preparation may be considered to be due to differences in the amounts of such contained. For example, of the

trypan blue sold on the market, there may be found small amounts of o-tolidine and H-acid which are the starting materials of synthesis. But the starting materials contained frequently in such products do not seem to have a connection with toxicity; the reason being that the trypan blue injected into rats is in the form of an aqueous solution and almost all the o-tolidine which is not soluble in water can be removed. As seen in Fig. 7 and 8, 1 per cent aqueous solution of trypan blue (Merck) did not contain the corresponding concentration of H-acid. Also when a 1 per cent aqueous solution of H-acid is injected every 2 weeks for 4 months into rats fed with DAB, there are seen no considerable differences in mortality when compared with control rats (Table VIII). As is clear from the photographs, Merck's trypan blue contains at least 3 varieties of dye, and these in the absorption spectrum, shown in Fig. 9 present slight differences in the wave lengths of their maximum absorption for their respective visible regions. At present we know nothing regarding the detailed chemical structure of these dyes, but when hydrogen peroxide is added to an aqueous solution of trypan blue and allowed to stand at room temperature for more than one day, or allowed to heat for about 3 hours in boiling water, the blue violet colour of trypan blue can, with the naked eye be seen to have changed into red. However, it was not possible to convert the red dye so obtained or similar coloured dye isolated with aluminium oxide into the principle blue coloured dye when the red solution were incubated for about 24 hours at room temperature in some reductants (1 per cent $NaHSO_3$ and 0.1 per cent $SnCl_2$). Experiments are now under way to find out which of

Table VIII. The Influence of H-acid on the Carcinogenesis induced by p-dimethylaminoazobenzene.

Experimental group	Number of rats		Averages of body weight (mg.)		Rats which died before the ends of experiment		
	at initial	at final	at initial	at final	Rat No.	Experimental days	A total dose of H-acid (mgm.)
H-acid group*	10	7	98.8	156.0	1	33	30
					2	66	50
					3	102	70
	4	3	109.3	128.3	1	103	80
DAB group**	16	15	139.0	39.0	1	66	—

* Rats received 10 injections of 10 mg H-acid and 0.06–0.09 per cent p-dimethylaminoazobenzene containing diets over 146 days.

** Rats fed with 0.06–0.09 per cent p-dimethylaminoazobenzene until a total dose of about 650 mg was ingested to each rat, but received no injection of H-acid.

The above experiments are now in progress, and at a period of about 5 months after the start of DAB feeding.

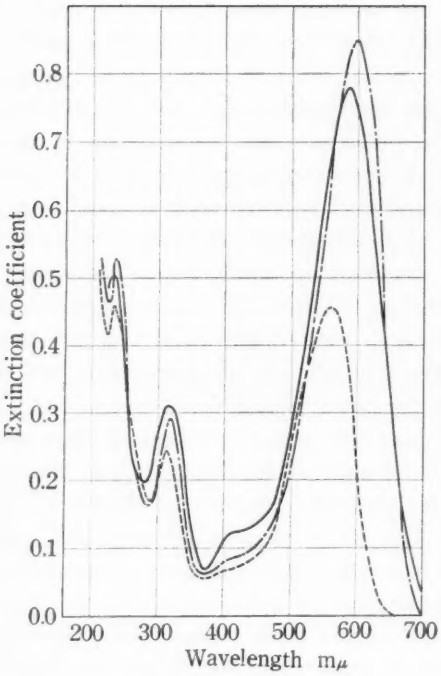


Fig. 9. Separation of the commercial trypan blue by use of a 1×30 cm. column of aluminium oxide (standerdisiert Al203 zur chromatographischen Adsorptionsanalyse nach Brockmann). test material; 1 ml of a 1per cent aqueous solution of trypae blue (Merck). eluting agent; distilled water as shown at flow rate of 1 ml per 4 minutes.

..... : 16–18ml of eluting agent through column.

—·—·— : 46–48ml of eluting agent through column.

——— : 0.005 per cent aqueous solution of trypan blue.

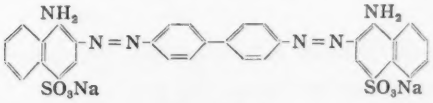
these dyes inhibit DAB carcinogenesis, and which is related to the toxicity of Merck's trypan blue. The results will be reported in the near future.

Discussion

It has been pointed out by many investigators that there exist between transplanted and spontaneous cancers marked differences in biological, biochemical and many other characteristics. In general malignant tumours seen in human beings are spontaneous primary tumours. Hence, in studies on tumours in animals, inhibition of carcinogenic processes and destruction of spontaneous cancer cells have presented important problems, since the discovery of carcinogenic substances. And up the present, from extensive biological studies the so-called protective diets and a few substances have been found that markedly inhibit carcinogenesis resulting from the feeding of azocarcinogens. In recent years Miller and others have studied the action mechanism of some of these inhibiting substances, and during the past few years many noteworthy reports have been made. Their views so far indicate that a few substances which inhibit carcinogenesis induced by DAB and its derivatives may be classified into the following; (1) substances that decrease the amounts of total protein-bound DAB, or delay the time required

for their arriving at a maximum; and (2) substances that are similar in entirety to the controls in the amount and in the speed of reaching a maximum of the bound DAB. It is not known whether changes that are seen in such protein-bound DAB are the cause or result of DAB carcinogenesis, but as malignant tumours are clearly produced by factors other than DAB, and similar azocarcinogens, it is natural that experiments be directed to substances that have marked inhibitory effects and yet do not produce any influence on the bound DAB. Fortunately, our experiments showed that trypan blue produces practically no effect on total protein-bound DAB. Hence, as far as this point is concerned it may be considered that the mechanism of liver cancer inhibition by this dye differs from that by riboflavin. But from similar experiments by Sayama and others (1954), the injections of trypan blue result in lowering of amount of protein-bound DAB which corresponds with a low incidence of liver cancer. However the values obtained by them are too small in both the amounts and differences of bound DAB. Hence, we believe that their conclusions are not proper. When we consider the inhibitory mechanims of DAB carcinogenesis, it is apparent that the problems of binding of the two dyes with liver protein become very important. In this respect the experiments with congo red are especially interesting. As may be shown in Fig. 10 this dye resembles in chemical structure trypan blue, and besides being bound to soluble proteins, the reaction whereby the dye is bound to the insoluble proteins, amyloids, is utilized in the diagnosis of amyloidosis (Lipstein, 1938). This amyloid, is a hyalinelike material, apparently of protein nature, that accumulates in the liver, kidney, spleen and adrenals during long-continued, infective, tissue destructive processes. Our experiments

Trypan Blue



Congo Red

with trypan blue also showed results that a fair amount of this dye is bound to the so-called insoluble components of the liver, probably proteins. In carcinogenesis characterized by a comparatively long continued process, nothing is known as to what role the above-mentioned insoluble constituents of the liver play. Further, the fact that trypan blue when injected into rats, leads to the formation of sarcoma, and yet has an inhibitory effect on DAB carcinogenesis is a pheno-

menon that resembles the striking observations of Richardson et al. (1952) and E. C. Miller et al. (1953) that the administration of 20-methyl-cholanthrene to rats receiving 3'-methyl-DAB markedly inhibit liver tumour formation. If the trypan blue used in our experiments is a single substance containing no impurities, then, when into the same animal a sarcoma producing and a cancer producing substance are introduced, they can be considered to act antagonistically. However, as has been stated here, Merck's trypan blue is not a pure product. At present investigations are under way to separate as quantitatively as possible the red, purple and blue dyes, from a 1 per cent aqueous solution of trypan blue (Merck), and to determine too, what factor is responsible for the inhibition of DAB carcinogenesis. Needless to say, this will also be connected with the differences in toxicity reported for various commercial products of trypan blue, as has been pointed by Simpson and others. If a dye component of the commercial trypan blue that induces a sarcoma is an anticarcinogen itself, what may be the picture be with congo red and trypan red, dyes that have failed according to Gillman et al. to produce tumours in rats. At present investigations are being made by us to test the efficacy of various substances that resemble trypan blue closely in chemical structure, and part of the work will be reported in the near future. Again, what action will this dye show against other spontaneous tumours. The results obtained by us regarding the biochemical interaction of trypan blue and DAB are only one step of a basic attempt to clarify these matters. There remain many problems that have to be solved.

Summary

The striking fact that the subcutaneous injections of trypan blue into rats inhibit liver tumor formation by p-dimethylaminoazobenzene (DAB), was studied particularly in regard to certain changes in the metabolic pattern and to the chemical nature of this inhibitory mechanism. Investigations were also made to determine which of the impurities present in Merck's trypan blue are responsible for the toxicity noted.

Regarding the enzymes (succinic dehydrogenase, cytochrome oxidase, catalase) and riboflavin subjected to determination, the livers of rats that received 60 mg of trypan blue and 600–700 mg of DAB for over 225 days showed in general a metabolic pattern which was significantly different from that seen in liver cancer, resembling though not perfectly, that in normal liver. However, there were seen some increase in hepatic desoxyribonucleic acid content, and some decrease in the activities of xanthine oxidase and d-amino acid oxidase, indicating a pattern which is intermediate between normal and cancerous livers.

Based on Schneider's and Dounce's methods, the trypan blue within the rat

liver was found to be bound to cellular components other than the nucleus, probably to the protein or proteins. But with the method described by Miller et al., trypan blue produces no considerable effect on the levels of the total protein bound DAB contained in the livers of rats within the period studied. Some attempts at analysis were carried out from the view points of electrophoretic analyses and other similar methods, and the results were discussed in relation to the enzyme-deletion hypothesis of cancer formation, but the ultimate cause of liver cancer inhibition still remains a matter for speculation.

The Merck product of trypan blue used in our experiments was not pure, but apparently contains at least, two or three types of dye. Also the starting materials for the synthesis contained frequently in the various commercial products do not seem to have a connection with the toxicity of a 1 per cent aqueous solution of trypan blue (Merck). There was no evidence as to which component of the commercial dye is responsible for the toxicity and inhibition of liver cancer induction.

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要　旨

ラッテ肝におけるトリパン青とパラヂメチルアミノアゾベンゼンの生化学的相互作用 (I)

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すでに，岩瀬，藤田，及び猿山，宮地らは，トリパン青がラッテによる実験的肝癌発生を著るしく抑制することを報告した。DAB による肝癌の抑制実験は多数報告せられ，その機構についても種々の論議がなされている。われわれはこれら2種の色素が何れもアゾ色素であることから，その抑制機構について，肝蛋白に対する2つの色素の結合の競り合いによるのではないかと仮定した。しかし，少くとも Miller 等の蛋白結合 DAB に対しては，トリパン青はほとんど影響を与えないことを知った。その抑制に関連して，カタラーゼ，リボフラビン，呼吸酵素，及び核酸の推移が検討されたが，最終の機構については，現在のところ如何なる解釈も与え得ない。

トリパン青はラッテに細網肉腫を作る。従って，この色素による肝癌発生の抑制は，まさしく 3′-メチル-DAB による肝癌発生を 20-メチルコラントレンが抑制した Richardson 等の知見に類似している。しかしわれわれが抑制実験に用いたトリパン青のメルク製品は，多くの他の製品と同様に不純物を含有している。すなわち，濾紙クロマトグラフィー，吸着クロマトグラフィー，濾紙電気泳動上，メルク製品は少なくとも3種色素赤，紫，青の混合物であった。従って，かかる製品を用いては，一方において細網肉腫を作る色素成分が，同一個体において直ちに他の発癌物質による肝癌発生を抑制するとは言い得ない。

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ON THE STRUCTURE OF METABOLITES OF CARCINOGENIC HYDROCARBONS*

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A considerable number of studies have been made in relation to the metabolism of carcinogenic hydrocarbons. Through these experiments the metabolic products of some of the polycyclic hydrocarbons have been isolated. It is a remarkable fact that in rats and mice the polycyclic hydrocarbons give rise to phenols in which the hydroxyl groups occupy the positions different from the points at which the oxidation would take place in a test tube. That is, 3: 4-benzpyrene is converted to 8-hydroxy-3: 4-benzpyrene (1, 2), 1: 2-benzanthracene to 4′-hydroxy-1: 2-benzanthracene (3), chrysene to 1-hydroxy-chrysene (4), and 1: 2: 5: 6-dibenzanthracene to 4′, 8′-dihydroxy-1: 2: 5: 6-dibezanthracene (7, 12, 18, 19). In order to explain the mechanism of such a metabolic oxidation various models have been postulated by many authors. No satisfactory explanation has been given, however.

Some of the present authors have introduced the **frontier electron method** (15, 16, 17) as one of the quantum-mechanical treatment of chemical reactivity. In the previous paper (20) the frontier electron method was put into application to the carcinogenic problem and an intimate correlation was pointed out between the carcinogenic activities and the frontier electron distributions in aromatic hydrocarbons. Furthermore, it is applied in the present paper to the problem of metabolism of carcinogenic hydrocarbons, in the search for an explanation of the structure of metabolites.

Result and Discussion

Fieser (9, 14) and Weigert (23) assumed that the carcinogen (for example 3: 4-benzpyrene) in rats and mice would be converted to 8: 9-dihydroxy-8: 9-dihydro-3: 4-benzpyrene by the addition of hydrogen peroxide and then transformed to 8-hydroxy-3: 4 benzpyrene by loss of water. On the other hand, Pullman (21, 22) postulated that the carcinogen (for example 1: 2-benzanthracene) would combine

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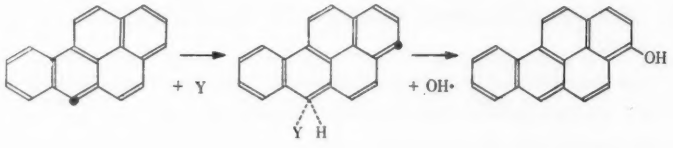
with tissue at K-region and 3′ : 4′-epoxide would be formed intermediately since that position of this tissue-carcinogen conjugate was found to be reactive to an electrophilic reagent. Bs enzymatic hydrolysis the epoxide would be converted to its trans-diol and then to 4′-hydroxy-1 : 2-benzanthracene by dehydration. In these two theories the diol has been considered as the precursor of phenol. Boyland and Wiltshire (6, 8), however, have recently stressed that the processes of phenol formation and diol formation are separate and independent of each other. In this connection the above-stated theories might deservedly be reconsidered.

Daudel (10, 11) postulated that the carcinogen, taking 1 : 2-benzanthracene as an example, would combine with tissue at K-region and in this tissue-carcinogen conjugate 4′ position would be most reactive, so that oxidation would take place at that position and 4′-hydroxy-1 : 2-benzanthracene would be formed. In that paper, however, the calculation was carried out only for the cases of benzene and naphthalene and no quantitative treatment was made for other polycyclic hydrocarbons. Pullman and Baudet (21) pointed out that according to their calculation based on Daudel's model the position which should be theoretically most reactive is not coincident with the position of attack in experiment.

The present authors postulate a model for metabolism of polycyclic aromatic hydrocarbons and will show how the structure of the metabolites formed is explained according to this model. The carcinogen, for example 3 : 4-benzpyrene,



(I) (II) (III)

Chart I.-A model of metabolic oxidation of carcinogenic hydrocarbons. Black spot in (I) indicates the position which is most reactive to electrophilic reagents. (Y) represents a certain kind of enzyme which here acts as an electrophilic reagent.

3:4-BENZPYRENE
(20π-ELECTRONS IN 21π-ORBITALS)

1:2:5:6-DIBENZANTHRACENE
(22π-ELECTRONS IN 24π-ORBITALS)

Chart II.-A theoretical interpretation of the electronic structure of carcinogen-enzyme conjugate ((II) in Chart I). The mode of π-conjugation is shown schematically by dotted lines.

would interact with some group, possibly with an enzyme (Y) forming a weak bond at the most reactive 5 position, as shown in Chart I. The electronic structure of this conjugate might be entirely different from that of the original hydrocarbon and is assumed to be in the calculation equivalent to such a model as shown in Chart II. There the most reactive meso-position is attacked by the enzyme, which is here considered as an electrophilic reagent, and the π-electrons are delocalized from the aromatic nucleus to the pseudo-π-orbitals (15, 20) through the loose bond which might be formed between the hydrocarbon and the enzyme. Thus, these pseudo-orbitals act as π-electron acceptors, and the degree of the electron migration can be varied by the value of exchange integral between meso-carbon and the pseudo-orbital. Calculated results show that in this case the qualitative prediction of the position of reaction is not so seriously affected by the value of exchange integral. The present authors consider the conjugation stated above to be very small and take the limit where the exchange integral tends to zero. The Coulomb integral of the pseudo-orbitals is taken as $\alpha+a\beta$ (20) where a may be arbitrary so long as $a \geq 1$. The frontier electron distribution (20) is calculated according to this model. The results of calculation show that 8 position (indicated by a black spot in (II)) is to be most reactive to a radical reagent in the case of 3, 4-benzpyrene. Accordingly the substitution by OH radical

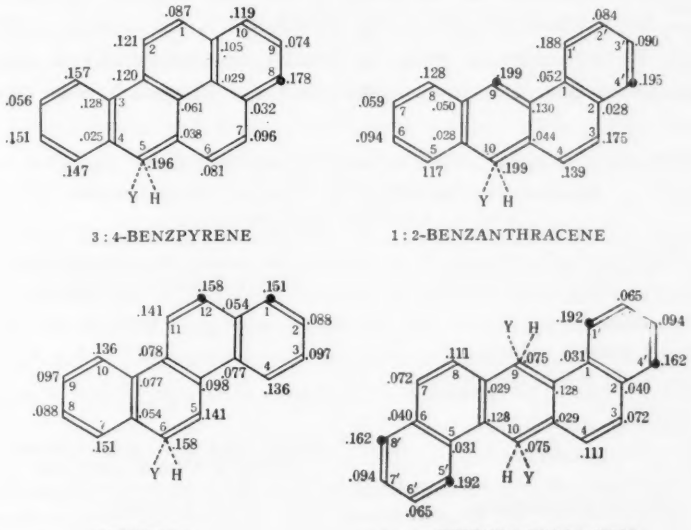


Chart III.–The frontier electron distribution of carcinogen-enzyme conjugates. Numerical values indicate the frontier electron density at each position in the conjugate, and the position where the density is markedly large is shown by a black spot.

would take place at that position, resulting in the formation of 8-hydroxy-3 : 4 benzpyrene. The positions of hydroxyl group in other metabolites are explained in the same way. The calculated results of the electronic structure of the carcinogen-enzyme conjugates are shown in Chart III. The numerical values represent the frontier electron density for a radical substitution at each carbon atom, and the larger the value is, the more reactive is that position.

From Chart III it is clearly understood that the metabolic oxidation takes place at 8 position in 3 : 4-benzpyrene and at 4′ position in 1 : 2-benzanthracene. In 1 : 2 : 5 : 6-dibenzanthracene, the frontier electron densities at 4′ and 8′ positions are a little smaller than the values at 1′ and 5′ positions where the attack of metabolic oxidation has not been reported. This may be attributed to the steric hindrance there. As for chrysene, the values of frontier electron density at 12 and 1 positions are large and almost equal to each other. Therefore, the expected structures are 12- and 1-hydroxy-chrysenes among which only 1-hydroxy-chrysene has already been found as a metabolite. Thus, a nearly satisfactory explanation has been given as to the structure of metabolites of polycyclic hydrocarbons.

In Table I, the value of frontier electron density at the principal carcinogenophore (20) of each original hydrocarbons is compared with that of its metabolite. For the original hydrocarbons these values have previously been reported (20) and the values for metabolities are calculated in the same manner. In the course of calculation the Coulomb integral at OH substituent is put equal to α (16, 20). It is seen in Table I that the value of frontier electron density at the principal carcinogenophore decreases remarkably when the hydrocarbon is metabolized.

Table 1. Comparison of the Values of Frontier Electron Density at Principal Carcinogenophore of Polycyclic Hydrocarbons with the Values of Their Metabolites.

Original Hydrocarbons	The Value of Frontier-Electron Density at Carcinogenophore
3 : 4-Benzpyrene	0.3025
1 : 2-Benzanthracene	0.3435
1 : 2 : 5 : 6-Dibenzanthracene	0.3714
Chrysene	0.4100
Metabolites	**The Value of Frontier-Electron Density at Carcinogenophore**
8-OH-3 : 4-Benzpyrene	0.1593
4′-OH-1 : 2-Benzanthracene	0.2138
4′, 8′-OH-1 : 2 : 5 : 6-Dibenzanthracene	0.2471
1-OH-Chrysene	0.3728

This result coincides with the well-known fact that the carcinogenicity of polycyclic hydrocarbons is lost or otherwise considerably weakened by the metabolic oxidation. Hence, it may be said that the metabolic oxidation is considered as a process of detoxication. Such a consideration has already been made by Fieser (9) and Dobriner et. al. (13).

If a somewhat speculative discussion is here permitted, the following will be added. There seems to be almost no doubt about the fact that the principal carcinogenophore plays the main role in the course of carcinogenesis. On the other hand, the chemists do not hesitate to recognize that in ordinary chemical reactions the most reactive position is not the principal carcinogenophore but the meso-position in such condensed aromatic hydrocarbons. In the tissue there might exist electrophilic reagents of various sorts and there is no reason to believe that the meso-position is attacked by none of them. It is the opinion of the present authors that the very reaction which is due to the chemical reactivity of meso-position is the combination of carcinogen with tissue at that point, which causes the detoxication, and, moreover, some of the carcinogen molecules which do not come into contact with detoxicating reagents would be subjected to a chemical combination with a certain kind of protein at the principal carcinogenophore, which might become the cause of tumor production.

Summary

1. Frontier electron method, one of the quantum-mechanical treatment of chemical reactivity, is applied to the problem of metabolism of carcinogenic polycyclic hydrocarbons and a nearly satisfactory explanation is given as to the well-known fact that the metabolic oxidation of these hydrocarbons takes place at the position different from the point at which oxidation occurs in a test tube.

2. The frontier electron densities at the principal carcinogenophore of the metabolities such as 8-hydroxy-3 : 4-benzpyrene, 4′-hydroxy-1 : 2-benzanthracene, 1-hydroxy-chrysene and 4′, 8′-dihydroxy-1 : 2 : 5 : 6-dibenzanthracene are obtained. They are much smaller than the values of the original hydrocarbons. This coincides with the experimental result that the carcinogenic activity of polycyclic hydrocarbons is lost or otherwise considerably weakened by metabolic oxidation.

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要　旨

発癌性炭化水素の代謝産物の構造について

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1. 発癌性芳香族炭化水素の代謝産物は，通常の化学反応における反応様式からは，ほとんど予測できない構造を有している。すなわち，酸化をうけた位置が，通常の試験管内酸化実験で期待される反応位置といちじるしく相違している。この事実を説明するために，いろいろの理論が提出されたが，いずれも不充分なもので，しかも代謝産物のすべてについて共通に説明を与えうる理論は見当らない。

著者らは，さきに芳香族炭化水素の化学反応性を説明する理論として，フロンテイアー電子法を提出し，これによって非置換芳香族炭化水素の発癌性についても満足な説明を与えることができたが，さらにこれら炭化水素の代謝過程にたいしてある仮定を置くことにより，8-オキシ-3,4-ベンツピシン，4′-オキシ-1，2-ベンツアンスラセン，1-オキシクリセン，4′，8′-ジオキシ-1，2，5，6-ジベンツアンスラセンなど，いままでに構造の決定されているフエノール性代謝産物のすべてについて，その生成の理由をほぼ満足に説明することができた。

2. これら代謝産物の主発癌団（発癌反応における反応位置と考えられる）のフロンテイアー電子密度は，もとの化合物のそれにくらべていちじるしく低下していることがわかった。これは，発癌性化合物が代謝産物に変化すると，発癌性は失なわれるか，あるいはいちじるしく低下するという実験事実と一致する。このことから，従来一，二の研究者によって考えられているように，発癌性化合物の代謝を一つの解毒過程と考えた。　(文部省科学研究費による)

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FURTHER STUDY ON THE DISTRIBUTION OF NUCLEIC ACIDS IN THE TUMOR CELLS OF THE MTK-SARCOMA III

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It has generally been accepted that the nucleolus contains ribonucleic acid (RNA) and protein rich in diamino acids (Brachet 1942, Caspersson 1950, Davidson and Waymouth 1946, Davidson, Leslie and White 1948, Gersch and Bodian 1943, Kaufmann, McDonald and Gay 1951). Vincent (1952) expressed a different view, based on the chemical analysis of nucleoli isolated from starfish oocytes, that the bulk of the nucleolus was phosphoprotein. Fujii (1954) maintained that the nucleolus contained zinc playing a significant role in cell division in addition to other substances.

Regardless of the chemical nature of the nucleolus, a considerable amount of work has been done on the morphology of that body; some studies concern the distinction between the true nucleolus and the chromosome nucleolus, some others deal with the relation between the nucleolus and chromosomes, and so on (Biesele 1944 a, b, Darlington 1932, Davidson 1947, Gates 1939, 1942, Borysko and Bang 1951). Further, there have appeared some papers claiming synthetic activity of the nucleolus (Bradfield 1949, Caspersson 1950).

Working on the histochemical nature of the tumor cells of the MTK-sarcoma III, an ascites tumor of rats, after the application of the Feulgen reaction, the present author has found evidence that the nucleolus shows a positive response to the Feulgen reaction, due probably to the presence of some chromatic substances which exist adhering the nucleolus. Data favourable to this view have been reported by Davidson (1947) showing that there is an outer zone of Feulgen positive material in the nucleolus of mammalian liver cells. A similar feature has been shown by the present author to occur in the nucleolus of rat liver cells, demonstrating that there is a distinct border between the nucleolus-associated chromatin and the nucleolus (Hori 1956). Further, the author has encountered evidence suggesting that the neoplastic cells are not identical in detail with the

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ordinary tissue cells in this respect. The present study was undertaken in an attempt further to clarify the relationship in question between the nucleolus-associated chromatin and the nucleolus in tumor cells.

Material and Methods

The MTK-sarcoma III, an ascites tumor of rats, provided the material for this study. The sectioning of tumor cells was made following the method described in the former paper (Hori 1956).

The Feulgen reaction was subjected to the modification of Stowell (1945). The staining with toluidine blue was made according to Lison's method (1936). The methyl green pyronine technique according to Brachet (1940) was adopted. Both methyl green and pyronine were refined after Shibatani's method (1950) previous to the preparation of the Unna-Pappenheim solution. Ribonuclease produced by the Nutritional Biochemical Corporation was employed.

Two slides, one as control and the other as experimental, was always prepared and treated under the same conditions throughout the entire course of the experiments.

Observations

Feulgen reaction for desoxyribonucleic acid (DNA): The preparations after the application of the Feulgen reaction showed the nucleolus with a covering of red color. The covering looked like a corona, being diffusive in appearance. There was no clear border line between the nucleolus and the nucleolus-associated chromatin in most cases, although, in a few cases, the occurrence of the border line between the two substances was evident rather distinctly.

Toluidine blue method for RNA: The application of 0.1% toluidine blue solution to sections has shown the RNA locality in the nucleolus and the chromatin in nucleus, both being stained blue; the stainability was reduced after the use of the 0.2% solution of ribonuclease. But, there is a considerable difficulty in distinguishing morphologically the nucleolus-associated chromatin from the nucleolus, since they stained *en bloc*.

Methyl green-pyronine stain for nucleic acids: In order to differentiate the two substances, probably different in their chemical nature, the staining method with methyl green-pyronine was applied to the sections. By staining the sections with Unna-Pappenheim's combination method with methyl green and pyronine, the differentiation of the nucleolus-associated chromatin was successful: the nucleolus was stained as a whole with pyronine in bright red color, whereas the nucleolus-associated chromatin appeared as green threads, without showing a corona-like structure like that seen in the Feulgen test. Thus the results favour the view that there is a distinct boundary between the nucleolus and the chromatin in question, and that the two substances do not mix with each other. The situation is not similar to that already shown by the Feulgen reaction: with methyl

green-pyronine the nucleolus-associated chromatin was stained green as threads adhering the surface of the nucleolus, while with Feulgen stain the corresponding substance was stained diffusely in the periphery of the nucleolus. To bring out the evidence more clearly, the following experiment was undertaken.

Feulgen reaction with light green stain: When Feulgen stain was combined with light green, the nucleolus generally appeared as a reddish-green body in most cases. Exactly speaking, the body which was composed of the nucleolus and the nucleolus-associated chromatin, was stained greenish-red in its peripheral part and green in its central part. It seems probable from this fact that the nucleolus-associated chromatin mixes with the nucleolar material in the outer layer of the body.

Nucleolus at late prophase: Late prophase would be a significant phase in mitosis because then the nucleolus itself appears as an almost independent body, with no slight connection with the chromatin. In fact, the nucleolus at late prophase showed bright green in Feulgen stain with light green, while it was bright red in the stain with methyl green-pyronine. Further, there is no chromatic element around the nucleolus. It seems apparent that at late prophase the nucleolus is of a true nucleolar nature in both morphological and cytochemical respects, since there is no association between the nucleolus and the chromatin in question.

Discussion

(1) On the staining methods used in this study

Methyl green-pyronine stain: Brachet (1940) adopted the Unna-Pappenheim combination method with methyl green and pyronine to identify RNA with the aid of ribonuclease. The selective stainability of RNA with pyronine was indicated by the impairment of stainability after treatment with purified ribonuclease. The specificity of the methyl green-pyronine stain for nucleic acids has been examined by many workers (Kaufmann, McDonald and Gay 1951, Kurnick 1950, Lison 1936, Pollister and Leuchtenberger 1949, Shibatani 1949, 1951, Taft 1951, Chayen 1952). The results so far obtained from these studies show that RNA and depolymerized DNA are stained with pyronine and while highly polymerized DNA is stained with methyl green. In respect to the methyl green-pyronine method, Lison (1936) emphasized that the excellent value of the methyl green-pyronine stain lies in the selective detection of RNA. His emphasis was that the Feulgen reaction should be used for the discovery of the location of DNA.

Feulgen stain: The studies so far done on the specificity of the Feulgen reaction (Brachet 1947, Catcheside and Holmes 1947, Lessler 1953, Shibatani 1950) resulted in a striking agreement in that the Feulgen method is applicable as the

only reliable test for examination of DNA, when it is used under proper conditions.

Toluidine blue stain: The toluidine blue technique adopted in the present study is a method for RNA test devised by Lison (1936). The specificity of this method for RNA is secured by the simultaneous application of ribonuclease. In the present study the stainability of toluidine blue was impaired after treatment with ribonuclease that was used to efface the stainability of pyronine.

(2) On the nucleolus

Caspersson (1950) observed the histochemical nature of the nucleolus-associated chromatin and the composition of the nucleolus. He offered a discussion on the close relationship between the chromatin in question and the nucleolus. According to him, when cell growth starts the nucleolar material appears within the chromocenter, and then the latter is exploded by the former. Thus the main chromocenter becomes the main nucleolus-associated chromatin.

If the above view of Caspersson be correct, the morphological distinction between the nucleolus and the nucleolus-associated chromatin will not be clear, since these two substances occur in mixture. In such a situation, the cell would show the nucleolus which responses to the Feulgen reaction as a whole. This seems to be the case with most tumor cells observed.

Generally, the tumor cells of the MTK-sarcoma III contain a prominent and basophilic body—the so-called nucleolus in each nucleus. This body was stained with toluidine blue and its stainability was impaired by the treatment with ribonuclease. It appears therefore that it may contain RNA. The Feulgen reaction demonstrated the presence of DNA in the body in question, showing red coloration which is deep in the periphery of the body. After the application of the Feulgen reaction with light green, the body was stained greenish red in the peripheral part and green in the central portion. The results seem to suggest that the body is composed of two substances, DNA and RNA, so far as the nucleic acid test is concerned: RNA appears to be covered with DNA, and the border between the two is not distinct. The central part of the body was less colored with the Feulgen stain, but stained deeply with toluidine blue and light green. It may be the true nucleolus according to the view of Caspersson that the formation of the nucleolus occurs inside the chromocenter.

The nucleolus of rat liver cells is somewhat different in this respect: there is an outer zone of Feulgen positive material in the nucleolus (Hori 1956). The outer zone is morphologically very distinct, so that the Feulgen negative body is easily distinguishable from the surrounding Feulgen positive element.

Based on the above consideration, it seems highly probable that the nucleolus, if it be defined as a Feulgen negative body, occurs only in a very close connection with the nucleolus-associated chromatin in actively growing tumor cells.

Then it is the true nucleolus which can be detected by the application of the Feulgen reaction with light green.

The results attained by the ordinary Feulgen method and methyl green-pyronine technique may possibly be explained as follows:

The substances stained with methyl green by the application of the methyl green-pyronine technique are apparently chromatin threads, whereas the corona-like substance which makes its appearance through the Feulgen method seems

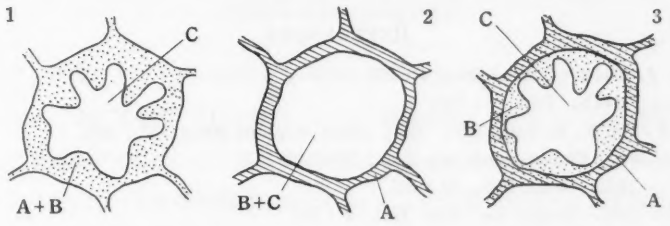

Fig. 1. shows outline marked by Feulgen stain; A and B indicate the substances in response to the Feulgen reaction.

Fig. 2. shows outline stained by methyl green-pyronine; A indicates chromatic threads stained with methyl green, and B and C the body stained with pyronine.

Fig. 3. shows the relation between A, B and C.

to contain certain elements in addition to the chromatin threads judging from its staining nature. Here the Feulgen positive substances in question can be divided into two components, "A" and "B". The chromatin thread component is referred to as "A". Attention should be focused on the substance "B". It is colored red in Feulgen stain, being not green in methyl green-pyronine stain. The staining nature of this substance strongly suggests that it may be depolymerized DNA, because of its non-staining with methyl green though there is no actual proof for this assumption (Figs. 1, 2, and 3).

Summary

By applications of the Feulgen reaction for DNA, the toluidine blue method for RNA and methyl green-pyronine technique for nucleic acids, the cytochemical relationship of the nucleolus and the nucleolus-associated chromatin was investigated in the tumor cells of the MTK-sarcoma III. From the results obtained it was suggested that; (1) In the rapidly growing tumor cells, the nucleolus occurs in very close connection with the nucleolus-associated chromatin, both cytochemically and morphologically. (2) The true nucleolus that is negative to the Feulgen reaction is probably surrounded by two somewhat different elements, highly polymerized DNA and depolymerized DNA. (3) The highly and depolymerized DNA, RNA and protein closely associated with one another to

constitute the so-called nucleolus which appears as a large prominent and basophilic body in the nucleus.

Acknowledgments

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要　旨

MTK-肉腫 III の細胞における核酸の分布に関する研究

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フォイルゲン反応，トルイヂン青による RNA の染色法及びメチルグリーン・ピロニン二重染色法を用い，MTK-肉腫 III を材料として，腫瘍細胞における仁と仁附随染色質の相互関係を細胞化学的に研究した。この実験の結果は次の事を示唆している様に思われた。すなわち (1) 急速に成長しつつある腫瘍細胞においては，仁と仁附随染色質とは，形態学的にも細胞化学的にも非常に密接な関係を有していて，両者を全く切り離して考えることは出来ない。(2) フォイルゲン反応に陰性な真性仁は，重合度の低い DNA よりなる層と，重合度の高い DNA よりなる層の二層によって取囲まれている。(3) ギムザ法，ヘマトキシリン・エオシン法等の染色法によってみられる仁は主として低重合及び高重合の DNA，RNA，蛋白質よりなっている。

(文部省科学研究費による)

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MONOAMINE OXIDASE ACTIVITY IN THE LIVER OF RATS FED ON HEPATIC CARCINOGEN

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So far as we know, no report has hitherto been published in respect to amine oxidase activity in the tissues of malignant tumors.

Of the enzymes of this category, in the first place, we brought monoamine oxidase into focus, introducing as substrates homologous aliphatic amines, namely, methylamine, ethylamine, propylamine, n-butylamine, n-hexylamine and n-heptylamine. At the outset determinations were made on the activity of this enzyme in normal rat liver. The essential part of this work, however, was made up of the studies of monoamine oxidase activity in the livers of rats fed on hepatic carcinogens, 4-dimethylaminoazobenzene (DAB) and 2-acetylaminofluorene (AAF), by using n-butylamine, n-amylamine and tyramine as substrates.

Materials and Methods

Experimental animals. Rats for prolonged feeding on carcinogenic diet were treated as follows: albino rats of male sex weighing about 100 g were kept on the diet containing DAB at the level of 0.06 per cent for 120 days or AAF at that of 0.05 per cent for 150–180 days. After the above duration of feeding the rats were removed from carcinogenic diet and were placed on the normal diet (rice grain alone) for additional number of days, i. e., 50–60 days for DAB fed rats and 90–120 days for AAF fed rats to avoid the direct influence of these chemicals to the enzyme.

When the rats thus treated were autopsied the livers showed already various grades of lesions, which were classified into four groups according to their grade, as a matter of convenience, namely, normal appearing liver (I), liver of uneven surface (II), cirrhotic liver (III), and the liver with hepatoma nodulus. This classification is symbolized in Tables and Charts with Roman numerals.

Furthermore rats fed on carcinogenic diet in their fourth week from the beginning of the experiment have also been employed. In these experiments the authors intended to see the enzyme activity of the livers, which have been exposed under

the direct influence of carcinogen and as yet showing no recogneizable hepatic lesion by macroscopic inspection.

Normal rats. Untreated male rats, which have been placed on normal diet, were used. All rats were given food and water *ad libitum* and green vegetables twice a week.

Enzyme preparation. The animals were sacrificed by cervical dislocation and exsanguinated by decapitation. The livers were then removed immediately and weighed. The homogenate was prepared in 0.1 M phosphate buffer at pH 7.8 and was exactly made up in dilution of 5 and 10 times of original fresh tissue with the buffer.

Estimation of monoamine oxidase activity. Oxygen uptake was measured at 37.5°C in an atmosphere of pure oxygen in Warburg manometric apparatus.

In the main compartment of the flask were placed 1 cc of 0.1 M phosphate buffer at pH 7.8 and 3 cc of homogenate corresponding to 0.6 g of wet tissue. In case of measurement of tyramine oxidase activity the constituents in the main compartment was changed because of its high activity as follows: 3 cc of 0.1 M phosphate buffer at pH 7.8 and 1 cc of homogenate equivalent to 0.1 g fresh tissue.

Into the side arm of the flask 1 cc of 0.01 M substrate (in case of hydrochloride, the solution has been adjusted beforehand to the same pH by using alkaline) was pipetted and tipped in after 15 minutes equilibration.

The control flask was made up similarly except neither substrate nor enzyme preparation was present, which had been replaced by the same amount of distilled water respectively.

Activity was calculated from the initial linear portion of the rate curve and expressed as oxygen uptake cmm per hour per mg of dried tissue homogenate.

For the measurement of tyramine oxidase activity another method has been employed to determine evolved ammonia during incubation. Into a large pyrex test-tube 1 cc of homogenate, which has been prepared merely in distilled water (equivalent to 0.1 g fresh hepatic tissue), 5 cc of phosphate buffer at pH 6, 7, 8 and 9, and 5 cc of aqueous solution of tyramine containing 5 mg substance and 4 cc of distilled water were delivered in succession and stoppered tightly.

After incubation at 37°C for 3 hours the liberated ammonia was transferred through aeration into a attached large pyrex test-tube containing 15 cc of 1/50 N H_2SO_4. Five cc of Nessler's solution was then added to 10 cc of the above H_2SO_4 solution and finally subjected to photocolorimetry against standard. The mixture of tissue homogenate without substrate and one with substrate and buffer solution but containing no homogenate were used as blank tests. The tyramine oxidase activity was represented in terms of ammonia-N γ per hour per 0.1 g wet weight of tissue.

Results and Discussion

Relative activity of monoamine oxidase in normal rat-liver has been determined by using 7 kinds of homologous aliphatic amines as substrates and was indicated in Chart 1. Among them, n-amylamine was most readily decomposed by homogenate. When its rate was taken as 100, those of n-butylamine, propylamine, n-heptylamine and n-hexylamine were 89, 56, 39 and 35 respectively, while methylamine and ethylamine were not decomposed at all by homogenate in the same condition. So far as these compounds concerned, the decomposition of substrate depends upon the number of carbon atoms in the molecule and C_5 compound was decomposed most readily. When the carbon atoms of substrate deviated from C_5, the digestibility was lessened.

Monoamine oxidase activity of livers of DAB and AAF fed rats were studied by using n-butylamine, n-amylamine and tyramine as substrates. Pathological but non-cancerous livers showed the activity as high as that of normal liver although somewhat lower than the latter according to the extent of developed lesions (Tables 1, 2 and 3; Charts 2, 3 and 4).

Table 1. Monoamine oxidase activity of livers of rats fed carcinogenic diet.
Substrate: n-butylamine

Normal rats	Carcinogen fed rats				
				DAB	AAF
1.20±0.35 (10)	Liver findings	Pathological but non-cancerous livers	I	1.08±0.13 (3)	0.96±0.10 (5)
			II	1.18±0.07 (4)	1.18±0.33 (4)
			III	0.83±0.09 (4)	0.61±0.25 (5)
		Hepatoma		0.43±0.10 (3)	—
	Duration of feeding: 4 weeks			0.54±0.17 (4)	0.62±0.01 (2)

Explanation for Tables 1, 2 and 3

Figures represent O_2 uptake in cmm / hr / mg dried tissue homogenate. Mean values with standard deviations and number of animals (in parentheses) for corresponding animals are shown in Tables 1, 2 and 3.

Roman numerals indicate the grade of hepatic lesions as they are described in text.

Livers of rats fed carcinogen for 4 weeks show usually normal appearance by macroscopic inspection.

Table 2. Monoamine oxidase activity of livers of rats fed carcinogenic diet.
Substrate: n-amylamine

Normal rats	Carcinogen fed rats				
				DAB	AAF
1.23±0.25 (8)	Liver findings	Pathological but non-cancerous livers	I	1.06±0.33 (4)	1.16±0.20 (4)
			II	1.20±0.10 (4)	1.19±0.26 (4)
			III	0.91±0.19 (4)	0.89±0.30 (5)
		Hepatoma		0.46±0.19 (2)	0.17 (1)
	Duration of feeding: 4 weeks			0.56±0.22 (3)	0.57±0.15 (4)

Table 3. Tyramine oxidase activity of livers of rats fed carcinogenic diet.

Normal rats	Carcinogen fed rats				
				DAB	AAF
4.32±0.61 (5)	Liver findings	Pathological but non-cancerous livers	I	3.83±0.63 (5)	3.57±0.56 (4)
			II	4.16±0.91 (7)	3.46±0.84 (3)
			III	4.84±1.10 (5)	3.90±1.50 (5)
		Hepatoma		2.17±0.68 (3)	2.91 (1)
	Duration of feeding: 4 weeks			2.84±0.77 (4)	2.57±0.34 (5)

When the liver once became neoplastic it showed markedly low activity. On that occasion the activity depended not only upon the sorts of carcinogens, which had been used for the feeding of rats, but also upon the kinds of substrates used for determination.

Liberated ammonia, while the digestion was going on, was estimated by distillation in case of tyramine oxidase determination and very akin results were obtained as those of manometric method. In this case, however, the activity in cirrhotic liver decreased markedly. Adjusting the digestion media at pH 6, 7, 8 and 9, it was confirmed that the pH optimum of tyramine oxidase was situated between 7 and 8 (Table 4).

Enzyme activity of livers of rats in their fourth week of DAB and AAF feeding experiment was also examined, and a marked depression was found compared to that of normal livers. It might be considered, that there was an inhibiting action

Table 4. Tyramine oxidase activity of rats fed carcinogenic diet. (measured by liberated ammonia during incubation)

			pH			
			6	7	8	9
Normal rats	Normal liver		46±18 (7)	118±20 (7)	122±12 (7)	106±8 (7)
DAB fed rats	Pathological but non-cancerous livers	I	50±6 (4)	112±22 (4)	118±18 (4)	98±20 (4)
		II	48±8 (6)	112±16 (6)	114±16 (6)	98±16 (6)
		III	36±8 (8)	84±22 (7)	82±18 (8)	68±16 (8)
	Hepatoma		10 (1)	30 (1)	36 (1)	28 (1)
AAF fed rats	Pathological but non-cancerous livers	I	46±14 (6)	106±20 (5)	102±16 (6)	82±16 (6)
		II	52±8 (13)	114±18 (13)	110±20 (13)	84±20 (13)
		III	44±16 (7)	90±14 (7)	84±20 (7)	68±10 (7)
	Hepatoma		22±4 (6)	42±16 (6)	38±14 (6)	34±10 (6)

Explanation for Table 4

Figures represent ammonia-N in γ / hr / 0.1 g wet weight.

Mean values with standard deviations and number of animals (in parentheses) for corresponding animals are shown. Roman numerals indicate the grade of hepatic lesions as they are described in text.

Chart 1 Oxidation of aliphatic amines (per cent) in the presence of monoamine oxidase (normal rat-liver homogenate) relative to n-amylamine

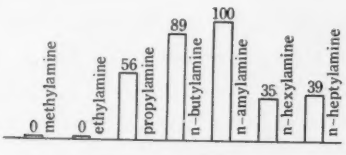
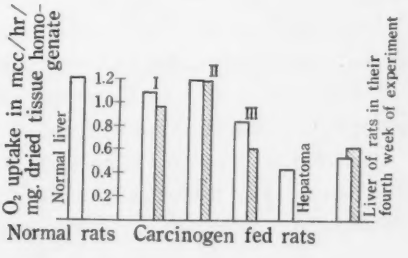


Chart 2 Activity of monoamine oxidase in liver of rats fed carcinogen

Substrate: n-butylamine



Explanation for Charts 2, 3 and 4

Mean values in Tables 1, 2 and 3 are illustrated in Charts 2, 3 and 4 respectively.

Unshaded bars in the right side of ordinate represent the activities of hepatic tissues of DAB fed rats and shaded bars represent that of hepatic tissues of AAF fed rats.

Roman numerals indicate the grade of liver lesions of pathological but non-cancerous liver as describe in text.

Liver of rats in their fourth week of experiment shows usually of normal appearance.

Chart 3 Activity of monoamine oxidase in liver of rats fed carcinogen.

Substrate: n-amylamine

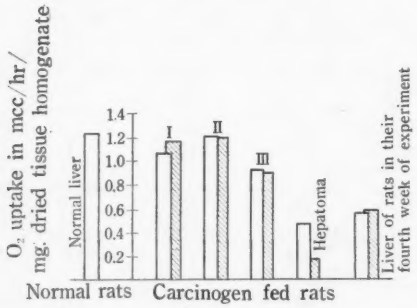
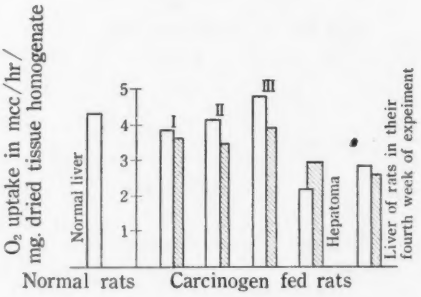


Chart 4 Tyramine oxidase activity in liver of rats fed carcinogen.



of carcinogens upon the monoamine oxidase action, because these livers have been standing just under the influence of the above chemicals (Tables 1, 2 and 3; Charts 2, 3 and 4).

Summary

1) Relative activity of monoamine oxidase in normal rat-liver was manometrically examined by using 7 sorts of homologous aliphatic amines as substrates. Among them n-amylamine was found to be the most readily decomposed substrate.

2) Activity of monoamine oxidase (n-butylamine, n-amylamine and tyramine were used as substrates) in livers of rats fed with carcinogens (4-dimethylaminoazobenzene and 2-acetylaminofluorene) for a long period of time, has been measured. Pathological but non-cancerous liver maintained the activity usually as high as that of normal liver, although in the marked cirrhotic liver the activity decreased somewhat. Hepatoma nodulus showed the activity less than that of normal liver.

3) Activity of tyramine oxidase was also measured by liberated ammonia and quite similar results as those of manometric determination were obtained.

4) In livers of rats in their fourth week of experiment of both carcinogens feeding, the activity has been depressed compared to that of normal liver, probably due to the direct influence of the carcinogens on the enzyme.

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要　旨

肝癌生成物質投与ダイコクネズミの肝モノアミン酸化酵素について

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ダイコクネズミにバターエロー及び2アセチールアミノフルオレンをそれぞれ長期間投与して後，正常食にもどしてなお飼育をつづけた動物の肝を実験に供した。これは肝癌にいたるあらゆる病変を持ち，しかも発癌剤の直接の影響のない肝を得るのが目的であった。

別に投与実験初期4週のネズミ肝を材料とした。これは発癌剤の作用下にあってしかも病変は肉眼的に認められない肝である。

酵素源として上記動物の肝及び対照として正常無処置動物の肝の均質液を用いた。基質はノルブチールアミン，ノルアミールアミン及びチラミンで燐酸塩緩衝液 (pH 7.8) と酵素のもとで検圧計により測定し，その酸素消費量により活性度を表した。

発癌に至らない病変肝は酵素活性度は正常肝と余り変らないが病変の進行に伴う低下の傾向がみられる。肝癌そのものは正常値の半分以下にも減ずる。この関係は投与した発癌剤の種類や測定に用いた基質の種類に拘らず認められる。

チラミン酸化酵素の場合測定法を換えて発生アンモニア量から活性度をみたが同酵素の検圧計によって得た関係と一致した。

投与実験開始4週後のネズミの肝は正常肝より活性度は低かった。投与発癌剤は肝の本酵素に抑制的に作用するものと思はれる。

なお脂肪族アミン7種を基質に選び正常肝について相互に比較したがノルアミールアミンが最も易く酸化された。

(厚生省厚生科学研究費による)

[GANN, 47, 231~236; June, 1956]

CHANGES IN THE GLUTAMINASE ACTIVITY OF LIVER TISSUE FROM RATS DURING THE DEVELOPMENT OF HEPATIC TUMORS BY CARCINOGEN FEEDING

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(Director: Prof. Sanji Kishi)

In connection with the studies of liver asparaginase activity during the development of hepatic tumors of rats fed with carcinogen, the present author took up this time the problem of glutaminase referring to former reports on the enzymes from our laboratory. These enzymes have been already investigated by Errera and Greenstein and their discovery of activating effect of pyruvate on these enzymes in all homologous hepatic tissues of rats, namely, resting adult liver, regenerating liver, fetal liver, and hepatoma was remarkable.

Materials and Methods

Experimental animals: Male albino rats of our laboratory stock were employed for experiments. The rats were fed with carcinogenic diet containing at the level of 0.06 per cent of 4-dimethylaminoazobenzene (DAB) for the duration of 120 days, and 0.05 per cent of 2-acetylaminofluorene (AAF) for 150–180 days respectively. After that, the carcinogen feeding was discontinued and the rats were placed on the basal diet (rice grain) without carcinogen for additional 50–60 days (in DAB rats) or 90–120 days (in AAF rats). When these rats were autopsied, the livers showed already various grades of lesions. They were grossly classified into two groups, namely, pathological but non-cancerous liver and hepatoma, and the former were subclassified into further three, I, II and III, according to their increasing grades. These Roman numerals were used in Table 1 and Charts 1 and 2.

Livers of rats fed on these two sorts of carcinogens in their fifth week from the beginning of experiment were also employed for measurement. In this case livers showed usually normal appearance by macroscopic inspection.

Normal rats: Male rats fed with basal diet were used. Food and water intake for all rats (both in normal and those placed on carcinogen feeding) was *ad libitum* and green vegetables were supplied twice a week.

Enzyme preparation: The hepatic tissues used for enzyme studies were excised from the animals immediately after killed by stunning. Samples of tissues were then blotted on paper, weighed, and homogenized and diluted with distilled water

exactly to 5 times of its original wet weight.

Determination of glutaminase activity: Determination has been carried out in our laboratory after the principle of Archbald's method as follows: 2 cc of hepatic tissue homogenate (equivalent to 0.4 g of fresh tissue) and 3 cc of phosphate buffer (pH 8) were placed in a flask of about 100 cc capacity with a long neck, and was stored at 38°C for 15 minutes before adding 2 cc of 0.1 M L-glutamine* solution, which has previously been kept at 38°C for 15 minutes.

After mixing these solutions, the flask was stoppered and kept at 38°C for exactly 30 minutes, then the digestion was stopped by addition of 1 cc of bromosulfalein solution (0.1 per cent) and the flask was at once immersed in ice water, stoppered tightly, and kept there until the ammonia was distilled. The blank consisted of the same amount of fluids, except glutamine solution which was replaced by 2 cc of water.

2 cc of saturated borate buffer (pH 10.1) was then added to the solution mixture in the flask, which was kept at 50°C preparing for the distillation procedure. Water vapor together with ammonia was distilled into a connected large pyrex test-tube containing 20 cc of 1/50 N H_2SO_4, with the aid of suction-pump under current of washed air.

The evaporation of the content in the flask was finished within 10 minutes, yet the aeration was carried out for 5 minutes more successively, after which the H_2SO_4 was delivered into a 200 cc volumetric flask and 5 cc of which was nesslerized. Finally it was subjected to photocolorimetry and measured against similarly treated standard solution of $(NH_4)_2SO_4$.

The glutaminase activities were then represented in two ways namely, ammonia-N γ / 0.4 g wet weight / 30 minutes, and ammonia-N γ / mg dried tissue homogenate / 30 minutes.

Results and Discussion

All the results were summarized in Table 1 and the mean values were represented graphically in Charts 1 and 2.

Activity of glutaminase in the pathological liver, including hepatoma, of DAB fed rats increased more than that of normal liver. There was no distinct relationship, however, between the grade of liver lesions and increase of glutaminase activity. Hepatoma itself showed a high activity calculated in its wet weight and extremely high in dry weight.

In the case of livers of AAF fed rats the results were not perfectly coincident with that of DAB fed rats. The slightly changed livers of AAF rats showed the activity nearly twice that of normal liver and higher than comparably affected

* General Biochemicals, Inc., Laboratory Park, Chagrin Falls, Ohio.

Table 1. Glutaminase activity in livers of rats fed on carcinogenic diet.

Normol rats		Carcinogen fed rats						
					DAB		AAF	
(1) 40.2 66-29.5 (10)	(2) 0.369 0.58-0.22 (10)	Liver findings	Pathological but non-cancerous liver	I	(1) 51.3 83-32.3 (5)	(2) 0.449 0.72-0.31 (5)	(1) 83.4 150.5-37 (5)	(2) 0.826 1.38-0.30 (5)
				II	57.0 96.5-34.0 (5)	0.526 0.72-0.34 (5)	79.8 100.5-54.5 (5)	0.875 1.30-0.55 (5)
				III	44.3 70.5-33.5 (5)	0.429 0.68-0.26 (5)	35.5 77.0-20.5 (5)	0.615 1.16-0.30 (5)
			Hepatoma		55.9 76.5-35.3 (4)	0.921 1.06-0.61 (4)	30.2 43.5-12.0 (3)	0.441 0.70-0.15 (3)
		Duration of feeding: 5 weeks			58.0 95.0-39.5 (5)	0.658 1.11-0.69 (5)	21.0 27.0-17.7 (5)	0.247 0.30-0.21 (5)

Figures, mean value with maximum and minimum values, represent ammonia nitrogen in γ, which was evolved from glutamine by enzyme preparation corresponding to 0.4 g fresh tissue, at 38°C for 30 minutes in columns (1), and to 1 mg dried tissue homogenate at 38°C for 30 minutes in columns (2).

Figures in parentheses represent the number of experiments for the determination of the corresponding data.

Chart 1 Liver glutaminase activity in rats fed with hepatic carcinogens.

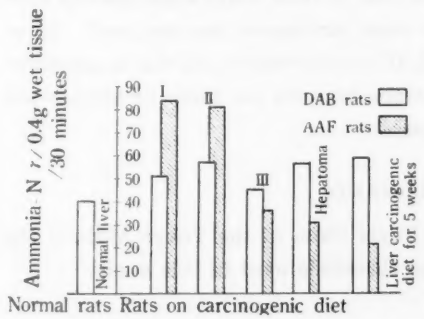
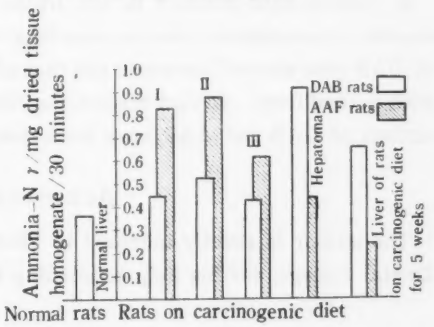


Chart 2 Liver glutaminase activity in rats fed with hepatic carcinogens.



livers of DAB fed rats. Hepatoma induced by AAF showed as high activity as normal liver and much lower than that of hepatoma produced by DAB.

The reason why there was slightly different results between AAF and DAB-hepatoma could not be elucidated at present, because the hepatoma contained no

more carcinogen, either DAB or AAF, even in trace.

Livers of DAB fed rats in their fifth week of the experiment showed a marked glutaminase activity and still no sign of inhibition was observed. Whereas the livers of AAF rats in the similar experiment showed more diminished activity than that of normal liver in regard to both wet and dry weight. It might be considered, that the livers of thus continuously fed rats were under the direct effect of carcinogen. So AAF might have acted, unlike DAB, as an enzymatic inhibitor against liver glutaminase. The difference between the mode of biochemical actions of hepatic carcinogens, AAF and DAB, could have been brought now to light in the early feeding experiment.

In connection with the above mentioned results attention may be called to our previous papers on asparaginase, in which we showed that its activity was depressed by both carcinogens similarly, when the rats were fed with them continuously for 3–4 weeks. This finding might be added as showing one of the different characteristics between asparaginase and glutaminase.

Summary

1) Liver glutaminase of rats fed on carcinogens, 4-dimethylaminoazobenzene (DAB) or 2-acetylaminofluorene (AAF), were studied.

2) Pathological livers including hepatoma of DAB fed rats showed a higher activity compared with that of normal livers.

3) Slightly affected livers of AAF fed rats showed very high activity. The activity of hepatoma induced by AAF, however, was practically identical with that of normal liver.

4) Glutaminase activity in the livers of rats in their early experimental days showed a inconsistent results according to what carcinogen has been used. Liver of DAB rats showed increased and that of AAF rats decreased activity as compared with normal liver. In that respect the difference between the mode of biochemical actions of DAB and AAF have been discussed.

Acknowledgments

The author is greatly indebted to Prof. Kunio Oota, of the Tokyo Medical and Dental College, for his help concerning the chemicals used in this study.

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要　旨

肝癌生成物質投与ダイコクネズミの肝グルタミナーゼについて

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肝癌生成物質，4-ヂメチルアミノアゾベンゼン (DAB) あるいは 2-アセチルアミノフルオレン (AAF)，を長期間ダイコクネズミに経口投与し，投与を中絶してなお正常食で長期間飼育をつづけてその肝性組織を材料に選んだ。また上記投与実験初期 (5 週) の動物の肝も用いた。対照は正常食ダイコクネズミの肝である。

グルタミナーゼ活性度は燐酸塩緩衝液の存在で基質 L-グルタミンが組織均質液によって分解される遊離アンモニアを Archbald の方法にならって定量した。そして組織の新鮮量および乾燥量に対してそれぞれ算出して活性度の数値とした。

DAB 投与ネズミで肝癌を含めて病変肝は正常肝のグルタミナーゼ活性度より高かった。この際活性度の増しと病変度の進行との関係は明らかでないが，肝癌は高く，特に組織乾燥量に換算した場合いっそう顕著であることがわかった。AAF 投与ネズミの結果は上述の DAB 投与ネズミの場合と必しも一致しなかった。AAF ネズミ肝の病変の軽度のものは正常肝の活性度の 2 倍程度に高い値を示した。この点は DAB ネズミと同傾向であるが AAF ネズミ肝癌のグルタミナーゼ活性度は上述の DAB 肝癌と一致せず，正常肝と同程度にとどまった。

初期実験で投与継続中のネズミ肝のグルタミナーゼ活性度は，投与した発癌剤によって明かに反対の結果を得た。先づ DAB ネズミについてみると，DAB の直接の作用下にある肝でありながら正常肝のそれより高い。AAF ネズミでは活性度は低く正常値よりもなお下っていた。このことは等く肝癌生成物質でも AAF は DAB と異なり，肝グルタミナーゼの作用に関しては酵素毒と考える。

因みに単に DAB だけについても酵素の種類によっては抑制的に作用する例がある。それは著者ら既報の肝アスパラギナーゼについての実験でアスパラギナーゼの活性度は DAB 投与 3～4 週で正常値より急劇に低下して最低値に達するという事実である。

(文部省科学研究費による)

[GANN, 47, 237~242; June, 1956]

CHOLINESTERASE ACTIVITY OF LIVER AND BLOOD SERUM FROM RATS DURING THE DEVELOPMENT OF HEPATOMA BY CARCINOGEN FEEDING

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(Director: Prof. Sanji Kishi)

In a recent paper Viollier and Waser (1950) have been published that acetylcholinesterase activity in hepatoma induced in rats by the feeding with 4-dimethylaminoazobenzene (DAB) was four times higher than that of normal liver, as represented by CO_2 evolution per unit weight of protein. Besides, they have measured acetylcholinesterase activity of blood plasma of tumorous animal and noted higher activity than that of control rat. Afterwards Langemann and Kensler (1951) demonstrated in hepatoma of rat, which has been produced by feeding with 3'-methyl-4-dimethylaminoazobenzene, an increased acetylcholinesterase activity in general, though not always. Furthermore, they tried to differentiate the activity between the two types of cholinesterase, namely, "nonspecific" and "specific" by using bezoylcholine and acetyl-β-methylcholine as substrate respectively, and they have found that their activity of either or both types were increased in hepatoma.

The present author examined the cholinesterase activity of hepatic tissue of rat fed on hepatic carcinogens, DAB and 2-acetylaminofluorene (AAF), in the course of liver cancer production by using acetylcholine and acetyl-β-methylcholine as substrate, and also the acetylcholinesterase activity of blood sera of the experimental animals. The author also investigated the acetylcholinesterase activity of the rats in their early experimental days of the carcinogen feeding, to examine if the carcinogen feeding itself has an influence upon the enzyme action of the liver.

Materials and Methods

Experimental animals. Carcinogen fed rats. Albino rats of male sex weighing about 100 g were used at the beginning of experiment, and were maintained for a long period of time on the diet containing 0.06 per cent of DAB or 0.05 per cent of AAF. Duration of feeding of DAB rats was 120 days and that of AAF rats was 150–180 days. After that the rats were taken off from the carcinogenic diet and were placed on the normal diet (rice grain) for an additional 50–60 days (in DAB rats) or 90–120 days (in AAF rats). Rats fed on one or the other carcinogen

for 1–4 weeks were also employed.

Normal rats. Untreated male rats, which have been placed on normal diet, were used. All rats were allowed to consume food and water *ad libitum* and were supplied green vegetables twice a week.

Enzyme source. The rats were narcotized with ether and their blood was firstly collected by cardiac puncture and allowed to clot for serum separation.

The hepatic tissues were removed promptly after the above procedure and homogenized with Ringer solution to obtain the exact 10 times dilution of their wet weight. Blood sera were diluted similarly to 10 times of their original volume. These solutions were employed as enzyme sources in this study.

In case of rats, which had been fed on carcinogenic diet for long duration, the livers showed already various grades of lesions. So they were classified for convenience according to their grade into four groups, namely, normal appearing liver (I), liver of uneven surface (II), cirrhotic liver (III), and liver with hepatoma nodulus.

Sera were also classified after the liver findings of corresponding rats, from which blood sera had been obtained.

Determination of cholinesterase activity. CO_2 evolution was measured at 37.5°C in the atmosphere of gas mixture, consisting of 95 per cent N_2 and 5 per cent of CO_2, contained in the flasks of Warburg manometric apparatus. The main compartment of the flask contained 2 cc of liver homogenate diluted with Ringer solution at pH 7.4. When serum as enzyme source was introduced, 2 cc of its dilution with Ringer solution was pipetted. In the side arm of the flask was placed 0.4 cc of 2 per cent acetylcholine chloride solution, with has been prepared in Ringer solution and tipped in after 15 minutes equilibration. The control flasks were made up in the same way, except substrate and enzyme source being replaced by the same volume of Ringer solution respectively.

In case of using acetyl-β-methylcholine as substrate, 0.4 cc of 10 per cent of the chloride solution in Ringer was pipetted into the side arm and 3 cc of liver homogenate was placed in the main compartment.

Readings were taken for 15', 30' and 45' after the process has been started and the value at 30' was multiplied by 2 to obtain that of one hour.

The enzymatic activities of the extracts were calculated from the initial linear rates of CO_2 evolution per hour per dried hepatic tissue homogenate and in case of serum, evolution of CO_2 per hour per cc of serum and per mg of serum-protein.

Results and Discussion

When the rats were fed on carcinogenic diet for a long period of time, their livers showed certain pathological changes, including hepatoma. Moreover the

Table 1. Acetylcholinesterase activity in livers of rats fed on carcinogenic diet. After long experimental days the rats are removed from it and placed on the normal diet for additional days.

Normal rats	Carcinogen fed rats				
				DAB	AAF
2.02±1.03 (11)	Liver findings	Pathological but non-cancerous livers	I	2.02±0.49 (5)	2.51±0.41 (4)
			II	2.12±0.86 (4)	2.45±0.85 (6)
			III	2.97±0.68 (8)	1.98±0.47 (7)
		Hepatoma		23.86±15.16(5)	19.25±20.36(3)

Roman numerals represent the grade of hepatic lesions as they are described in text.

Table 2. Acetylcholinesterase activity in livers of rats fed on carcinogenic diet in their early experimental days.

Normal rats	Carcinogen fed rats*		
	Feeding days in week	DAB	AAF
2.02±1.03 (11)	1	2.04±0.67 (4)	2.26±0.66 (2)
	2	1.88±0.29 (2)	1.89±0.28 (2)
	3	1.81±0.31 (2)	2.02±1.04 (2)
	4	3.13±0.44 (4)	2.20±0.97 (5)

* Livers of the rats show usually normal appearance.

Table 3. Cholinesterase activity in livers of rats fed on carcinogenic diet by using acetyl-β-methylcholine as substrate.

Normal rats	Carcinogen fed rats				
				DAB	AAF
0.585±0.079 (5)	Liver findings	Pathological but non-cancerous livers	I	0.646±0.002(2)	0.886±0.011(2)
			II	0.450±0.198(3)	0.419±0.049(2)
			III	0.506±0.040(5)	0.485±0.249(6)
		Hepatama		1.468±1.195(3)	0.910±0.800(2)
	Duration of feeding: 4 weeks*			0.722±0.122(3)	0.459±0.128(2)

Roman numarals represent the grade of hepatic lesions as they are described in text.

* Livers of the rats in their early experimental days show usually normal appearance.

Explanation for Tables 1, 2 and 3

Figures represent CO_2 evolution in c mm / hr / mg dried tissue homogenate. Mean values with standard deviation and number of animals (in parentheses) for corresponding animals are shown in Tables.

Table 4. Acetylcholinesterase activity in blood sera of rats fed on carcinogenic diet.

Sera of normal rats				Sera of DAB fed rats		Sera of AAF fed rats	
(1)	(2)			(1)	(2)	(1)	(2)
3.90±1.09(11)	6.29±1.77 (7)	Rats having pathological but non-cancerous livers	I	3.23±0.98 (5)	5.05±1.81 (4)	2.75±0.30 (3)	4.05±0.77 (3)
			II	4.30±2.44 (4)	6.83±4.43 (3)	3.81±0.77 (4)	5.54±1.93 (3)
			III	4.14±0.95 (6)	5.62±1.00 (4)	3.42±1.10(11)	5.01±1.62(10)
		Hepatoma-bearing rats		4.46±1.52 (4)	8.52±3.40 (3)	3.52±0.59 (4)	5.46 (1)
		Rats at fourth week of feeding*		3.58±1.44 (5)	6.28±1.77 (5)	4.14±1.10 (5)	8.58±2.47 (5)

Roman numerals represent the grade of hepatic lesions as they are described in text.

* When the rats are autopsied livers show usually of normal appearance.

Figures represent CO_2 evolution in c mm/hr/cc serum in columns (1), and CO_2 evolution in c mm/hr/mg protein in columns (2).

Mean values with standard deviation and number of animals (in parentheses) for corresponding animals.

rats were kept off the carcinogenic diet for a while before the experimental use, for the purpose of avoiding any direct effect on enzyme of carcinogen. When the liver lesions developed within the limit of non-cancerous, the activity of acetylcholinesterase showed no marked difference from that of normal rat. As soon as liver turned into cancerous, it showed very high activity, though the extent varied widely. The result was akin to the previous observation of Langemann and Kensler. Some specimens of hepatoma showed indeed 20–25 times higher activity than that of normal liver, independent from the sorts of carcinogen, with which hepatoma had been induced (Table 1).

Activity of "specific" cholinesterase has been measured by using acetyl-β-methylcholine as substrate and the above mentioned result has also been demonstrated, although it was less active (Table 3).

Activity of livers of rats fed on carcinogenic diet for 1–4 weeks was also studied (Tables 2 and 3). These livers though being disposed under the direct influence of carcinogen, showed still normal appearance by inspection. No significant difference have been found between normal liver and liver of these experimental animals.

The acetylcholinesterase activity using blood sera as enzymatic source was measured (Table 4). Even in sera of tumorous animals, including the

rats having pathologically changed livers, the activity was nearly the same as that of sera from normal rats. These findings were inconsistent with those of Viollier and Waser, who have found in blood plasma of their hepatoma-bearing DAB fed rats very high activity of acetylcholinesterase.

The sera of rats in their fourth week of experiment showed a high activity in AAF rats but not in DAB rats. These findings may indicate that the modes of biological action of DAB and AAF differ from each other.

Summary

1) Rats were fed with carcinogens, 4-dimethylaminoazobenzene (DAB) or 2-acetylaminofluorene (AAF), and livers and blood sera were employed for the study of cholinesterase activity.

2) The liver cholinesterase activity was measured using acetylcholine and acetyl-β-methylcholine as substrates, and there was found that no significant difference between normal rat-liver and pathological but non-cancerous liver.

3) When the liver once turned into neoplastic, it showed extremely high acetylcholinesterase activity and fairly high cholinesterase activity when acetyl-β-methylcholine was substrate.

4) Livers of rats in their early experimental days of carcinogen feeding showed nearly the same oder of activity as that of normal liver.

5) Acetylcholinesterase activity in blood serum showed no noteworthy difference even in hepatoma-bearing rats, including rats having pathologically changed livers.

6) Serum of rats fed on AAF, at the end of the fourth week of experiment, showed high acetylcholinesterase activity but not in the case of DAB fed rats in the same condition.

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要　旨

実験的肝癌生成過程のダイコクネズミ肝及血清のコリンエステラーゼについて

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ダイコクネズミに肝癌生成物質バターエロー (DAB) 及び2・アセチールアミノフルオレン (AAF) をそれぞれ投与し，実験開始後4週までのネズミ肝及び血清を実験に供した。また長期間投与後，発癌剤投与を中絶し正常食でなお飼育をつづけた動物についても同様に実験した。

肝はリンゲルで均質液を作り酵素液とし，基質はアセチールコリン及びアセチール・ベータ・メチールコリンを選んだ。血清はリンゲルで稀釈したものを酵素液とし，基質はアセチールコリンのみを用いた。そしてすべて検圧計により発生炭酸ガス量を測定して活性度を表した。

DAB, AAF 投与4週までのネズミ肝について正常肝との差異は認められず，また発癌にいたらない病変肝と正常肝との活性度の著差もなかった。ただ肝癌のみが平均して極めて顕著に高い価を示した。剖検的に全く同様な肝癌でも個体差があって，そのあるものは極端に高く正常値の 25 倍 (基質：アセチールコリン) の活性を示した。

血清については肝癌を持っている動物でも正常血清と差異は明らかでなかった。投与実験4週の場合 AAF ネズミ血清は正常血清より活性度は高かった。しかして DAB ネズミ血清は正常血清と変りなかった。ここにおいて DAB と AAF の作用機序に差異のあることが考えられる。

(厚生省厚生科学研究費による)

[GANN, 47, 243~244; June, 1956]

EXPERIMENTAL PRODUCTION OF CARCINOMA IN MICE WITH CIGARETTE SMOKE TAR*

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The high incidence of cancer of the lip, tongue, and throat in men as compared to the infrequency of such cancer in women has frequently been attributed to persistent irritation of the epithelial surfaces by tobacco tar, to which men have been exposed to a much greater degree. Stimulated by these observations, several investigators have studied the action of tobacco tar and smoke in different species of animals (1). The use of various kinds of tobacco, the preparation of their tars by burning at different temperatures, and the employment of animals of different strains have led to varying results.

The recent interest in pulmonary carcinogenesis in relation to cigarette smoking (2, 3), and vehicular exhausts (4, 5) leads us to give an account of our own experiments in which we produced malignant growths in mice in high percentage by long continued external application of tobacco smoke tar.

METHODS

The cigarette tar used in the present study was supplied by Dr. E. L. Wynder. It was obtained from cigarettes (standard-brand cigarettes) smoked in a manner similar to human smoking as closely as practically possible. The resulting smoke was condensed in flasks immersed in dry ice. The tar was then dissolved in acetone (ratio 1 : 1) and kept in a regrigerator until used. Fresh cigarette smoke tar was prepared monthly.

Approximately 80 mg of the cigarette tar was applied with a brush 3 times a week to shaved skin in the interscapular region of the backs of 60 male Rockland Swiss albino mice (about 35 days old and weighing about 20 gm) for a period of 2 years. The animals were given food (Purina Laboratory Chow) and water *ad lib.*

RESULTS

Of 60 tarred mice, 44, lived from 1 year to 516 days (23 mice were alive on October 3, 1955), and 10 of them developed 1 to 3 papillomas, and 7 or 16% had

* This investigation was supported by grants from American Cancer Society and by the Damon Runyon Memorial Fund for Cancer Research.

squamous carcinomas.

During the next 65 days 3 new mice developed papillomas and 3 other new mice developed carcinomas. Lung metastases were found in one mouse. In other words, of 60 tarred mice, 44 lived from 1 year to 580 days, and 10 of them or 23% had cancer.

During the following 116 days 3 new mice developed papillomas and 2 other new mice developed carcinomas. In other words, of 60 tarred mice 44 lived from 1 year to 696 days, and 16 of them or 36% developed papillomas, and 12 mice or 27% had squamous carcinomas. This was verified by histologic examination of the tumors. Four mice were still alive on March 31, 1956, one with skin cancer, one with papilloma, and 2 others with no tumors. Thus our findings confirm the observation of Wynder, Graham and Croninger (6) that cigarette tar is a definite carcinogenic agent to mouse epidermis. The first papillomas appeared in a mouse painted with cigarette tar on the 191st day and the earliest cancer on the 320th day.

Summary

Prolonged painting of the skin of mice with condensed cigarette smoke prodcued 16 papillomas (36%) and 12 squamous carcinomas (27%) among 44 Swiss mice that lived from 1 year to 696 days.

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要　旨

紙巻煙草の煙のタールによる実験的癌腫生成

杉　浦　兼　松

(Sloan-Kettering 癌研究所実験化学療法部)

紙巻煙草の煙を濃縮して得たタールを長期間にわたってマウスの皮膚に塗布することにより，1 ケ年以上 696 日生存した Swiss マウス 44 例中 16 例 (36%) にパピローマが，12 例 (27 %) に扁平上皮癌が発生した。

微生物工学講座

予約募集中

〔予約期限＝7月20日〕

詳細内容説明送呈

全9巻

最新の進歩を中心とする微生物工学の一大体系

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微生物を応用する科学は近時飛躍的な発展をとげつつあり，酵素化学・微生物遺伝学・抗生物質の方面で次々と未知領域が開拓され，それに伴う工業も著しい発展を見せている。本講座はこの時世に鑑み，微生物利用の基礎学から微生物学研究法を最近の新技術をとり入れ，微生物工学全般の把握を十分ならしめんとした。

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